University of Alberta

Increased Accuracy to Detect Post-Prandial Lipemia in Subjects with Hyper-insulinemia and Diabetes by Measuring Apolipoprotein B48 Lipoproteins from Whole Plasma

by

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Abstract

Post-prandial dyslipidemia is associated with the development of atherosclerosis. The atherogenic nature of aberrant post-prandial metabolism has been attributed to the ability of chylomicron remnants to enter arterial tissue and become entrapped within arterial vessels. Thus, interest has grown in the use of chylomicron metabolism for the assessment of cardiovascular disease risk. However, only a handful of techniques are available for the detection of chylomicrons and standardized methods have yet to be established. Ultracentrifugation has been used to isolate a lipoprotein fraction that is rich in nascent chylomicrons. More recently, an anti-serum to apolipoprotein B48 has been raised as an exclusive marker of chylomicrons, allowing for the specific determination of chylomicrons directly from whole plasma. Therefore, the aim of this thesis was to contribute knowledge on how best to measure chylomicron metabolism, either from whole plasma or a triglyceride rich lipoprotein (TRL) fraction at density < 1.006 g/ml, in order to provide the most accurate interpretation of post-prandial lipemia. Study 1: Chylomicron concentrations, as measured from whole plasma and TRL fractions, were compared in a population with established post-prandial lipemia (hyper-insulinemia). Fasting and post-prandial chylomicron concentrations were significantly greater when quantified from whole plasma compared to TRL fractions in hyper-insulinemic men. Study 2: Whole plasma chylomicron concentrations were utilized to examine post-prandial metabolism in type 1 diabetes mellitus (T1DM). Subjects with brittle T1DM exhibited elevated plasma chylomicron concentrations in the fasting and post-prandial state compared to healthy controls, but not from TRL fractions. Collectively, the studies of this thesis have shown that the detection of chylomicrons from whole plasma provides greater sensitivity to detect potentially atherogenic remnant particles during conditions of either hyper-insulinemia or T1DM.

Table of Contents

Chapter 1: Introduction

1.1 Rational 1

1.2 Thesis Aim 5

1.3 General Hypothesis 6

1.4 Specific Objectives 6

Chapter 2: Literature Review

2.1 Lipoproteins
2.1.1 Introduction 7
2.1.2 Chylomicrons 8
2.1.3 Very Low Density Lipoproteins 9
2.1.4 Low Density Lipoproteins 10
2.1.5 High Density Lipoproteins 11

2.2 Chylomicron Metabolism
2.2.1 Introduction 13
2.2.2 Chylomicrons and Cholesterol Metabolism 13
2.2.2.1 Chylomicron Production and Secretion 13
2.2.2.2 Chylomicron Clearance 14
2.2.3 Chylomicrons in Atherogenesis 15
2.2.4 Chylomicrons and Clinical Studies of Coronary Artery Disease 17
2.2.5 Conclusion 18

2.3 Chylomicron Metabolism in Insulin Resistance and Type 1 Diabetes
2.3.1 Chylomicron Metabolism in Insulin Resistance 19
2.3.1.1 Introduction 19
2.3.1.2 Physiological Role of Insulin 19
2.3.1.3 Chylomicrons and Clinical Studies of Insulin Resistance 20
2.3.1.4 Chylomicrons and Clinical Studies of Overweight 22
2.3.1.5 Chylomicrons and Clinical Studies of Metabolic Syndrome 22
2.3.1.5 Chylomicrons and Type 2 Diabetes Mellitus 23
2.3.1.6 Mechanisms Underlying Abnormal Chylomicron Metabolism in Insulin Resistance 24
2.3.2 Chylomicron Metabolism in Type 1 Diabetes Mellitus 27
2.3.2.1 Introduction 27
2.3.2.2 Chylomicrons and Studies in Type 1 Diabetes Mellitus 27
2.3.3 Conclusion 28

2.4 Detection of Chylomicrons
2.4.1 Introduction 29
2.4.2 Triglyceride-Rich Lipoprotein Fractions 29
2.4.3 Indirect Measures 30
2.4.3.2 Remnant-Like Particle Cholesterol 30
2.4.3.3 Retinyl Esters 31
2.4.3.4 Breath Test 32
2.4.4 Direct Measures 33
2.4.4.1 SDS-PAGE 33
2.4.4.2 Enzyme-Linked Immunosorbent Assays 34
2.4.4.3 Immunoblotting 35
2.4.5 Conclusion 36

2.5 References 45

Chapter 3: Quantification of Chylomicron Remnants in Fasting Hyper-insulinemia

3.1 Introduction 56

3.2 Methods 57
3.2.1 Subjects 57
3.2.2 Achieving the Fed Steady State 58
3.2.3 Biochemical Analysis 58
3.2.4 Statistical Analysis 60

3.3 Results 60
3.3.1 Physical and Fasting Biochemical Characteristics 60
3.3.2 Fasting Apolipoprotein B48 Concentrations 61
3.3.3 Apolipoprotein B48 Concentrations in the Fed State 61
3.3.4 Small Apolipoprotein B48 Remnants in the Fed State 63
3.3.5 Apolipoprotein B48 Appearance in the Plasma 63
3.3.6 Ratio of Apolipoprotein B48 to Triglycerides in the Fed State 64

3.4 Discussion 66

3.5 Conclusion 68

3.7 References 69

Chapter 4: Apolipoprotein B48 Remnant Lipemia in Type 1 Diabetes Mellitus

4.1	**Introduction**	72
4.2	**Methods**	73
4.2.1	Participants	73
4.2.2	Study Protocol	74
4.2.3	Biochemical Analysis	76
4.2.4	Statistical Analysis	77
4.3	**Results**	77
4.3.1	Subject Characteristics	77
4.3.2	Response of Post-Prandial Insulin and Glucose	78
4.3.3	Response of Post-Prandial Triglyceride and Cholesterol	78
4.3.4	Post-Prandial Response of Apolipoprotein B48	80
4.3.5	Comparison to Triglyceride-Rich Lipoprotein Fraction	81
4.3.6	Correlation of Plasma Apolipoprotein B48 with Post-Prandial Measurements	82
4.4	**Discussion**	82
4.5	**Conclusion**	85
4.7	**References**	86

Chapter 5: Collective Discussion and Conclusion

5.1	**Collective Discussion**	88
5.2	**Future Directions**	91
5.3	**Collective Conclusion**	92
5.4	**References**	93

List of Tables

Table 2-1	Major classes of human plasma lipoproteins: some properties	8
Table 2-2	Summary of studies reporting chylomicron concentrations from retinyl esters	37
Table 2-3	Summary of studies reporting chylomicron concentrations from ^{13}C Breath Test	39
Table 2-4	Summary of studies reporting chylomicron concentrations from TRL fractions, SDS-PAGE & coomassie staining	40
Table 2-5	Summary of studies reporting chylomicron concentrations from ELISA	42
Table 2-6	Summary of studies reporting chylomicron concentrations from immunoblotting	43
Table 3-1	Physical and fasting metabolic characteristics of normo-insulinemic and hyper-insulinemic subjects	61
Table 3-2	ApoB48 response to feeding in a steady-state among hyper-insulinemic and normo-insulinemic subjects as determined from whole plasma apoB48 and TRL fractional samples	62
Table 3-3	Initial response to feeding from 0 to 6 hour before steady-state is reached in hyper-insulinemic and normo-insulinemic subjects as determined by whole plasma apoB48 and fractional (TRL) methods	64
Table 4-1	3-day food records of type 1 diabetes subjects and matched controls	75
Table 4-2	Fasting plasma metabolic characteristics of type 1 diabetes subjects and controls	78
Table 4-3	Spearman's correlation coefficients for apolipoprotein B48 and plasma insulin, glucose and triglyceride responses	82

List of Figures

Figure 2-1	Schematic diagram of chylomicron hydrolysis and clearance by receptor-mediated processes	15
Figure 2-2	Schematic diagram of the initiation of atherosclerosis: lipid deposition and entrapment in the vessel wall	17
Figure 2-3	Schematic diagram of aberrant chylomicron metabolism in insulin resistance / hyper-insulinemia	26
Figure 3-1	Apo B48 AUC of normo-insulinemic and hyper-insulinemic subjects	62
Figure 3-2	Apo B48 remnants in density > 1.006 g/ml	63
Figure 3-3	TG:Apo B48 ratio determined from whole plasma and TRL fractions	65
Figure 3-4	TG:Apo B48 ratio in normo-insulinemic subjects and hyper-insulinemic subjects	65
Figure 4-1	The post-prandial response in plasma insulin and glucose for type 1 diabetic subjects and controls following the intake of sequential meals	79
Figure 4-2	The post-prandial response in plasma triglyceride and cholesterol for type 1 diabetic subjects and controls following the intake of sequential meals.	79
Figure 4-3	The post-prandial associated plasma apo B48 response following the intake of sequential meals	81

List of Abbreviations

Apo	Apolipoprotein
AUC	Area Under the Curve
BMI	Body Mass Index
CAD	Coronary Artery Disease
CI	Confidence Interval
CVD	Cardiovascular Disease
ECAM-1	Endothelial Cell Adhesion Molecule-1
ECL	Enhanced Chemiluminescence
ELISA	Enzyme Linked Immunosorbent Assays
ER	Endoplasmic Reticulum
FFA	Free Fatty Acid
Hb_{A1C}	Glycosylated Hemoglobin Concentration
HDL	High Density Lipoprotein
HOMA-IR	Homeostasis Assessment Model of Insulin Resistance
HR	Hazard Ratio
iAUC	Incremental Area Under the Curve
LCAT	Lecithin Cholesterol Acyl Transferase
LDL	Low Density Lipoprotein
LPL	Lipoprotein Lipase
RE	Retinal Esters
RLP-C	Remnant-Like Particle Cholesterol
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TG	Triglyceride
TRL	Triglyceride-Rich Lipoprotein
VCAM-1	Vascular Adhesion Molecule-1

VLDL	Very Low Density Lipoprotein
WHR	Wait-To-Hip Ratio

Chapter 1: Introduction

1.1 Rationale

Cardiovascular disease (CVD) is the leading cause of death in Canada, accounting for at least 36% of all deaths and $21.7 billion in direct and indirect cost of illness (Heart and Stroke Foundation of Canada, 2007). Despite the decrease in CVD-related mortality between 1997 and 2003, the Heart and Stroke Foundation of Canada (2007) predicts that there will be 100,000 more hospitalizations per year due to CVD over the course of the next 10 years. Thus, early diagnosis and treatment of CVD are essential in reducing its substantial medical, economic, and social burden. By definition, CVD encompasses numerous conditions involving the heart, its associated blood vessels, as well as the system of arteries and veins throughout the body and the brain. However, the two most common forms of CVD are coronary artery disease (CAD) and cerebrovascular disease, which are caused by a disease known as atherosclerosis (Heart and Stroke Foundation of Canada, 2007).

Atherosclerosis is a chronic disease characterized by the thickening and stiffening of the arterial walls (see details in Section 2.2.3, Figure 2-2) (Luis, 2000). The first stage of the pathological process begins with a fatty streak, an intracellular accumulation of cholesterol and macrophages within the vessel wall (Libby et al., 2002). The delivery of cholesterol to the vessel wall is due to lipid carriers known as lipoproteins (section 2.1). Thus, it is thought that increasing arterial exposure to potentially atherogenic lipoproteins increases the arterial retention of lipid (Nordestgaard and Tybjaerg-Hansen, 1992). Lipoprotein entry through the vessel wall has been shown to occur via transcytosis (the vesicular transport from the plasma to the subendothelial space) and is limited to particles that have a diameter that can be accommodated by the transcytotic vesicles

(maximum 70-80 nm). Initially, intracellular deposition of cholesterol by lipoproteins is metabolized by macrophages and removed from the subendothelial space. However, as lipid accumulation increases, the functioning of macrophages shifts from protective to unregulated, resulting in the formation of "foam cells" and the maturity of the fatty streak (Libby et al., 2002). Over time, the fatty streaks progress to mature plaques, consisting of foam cells, collagen, lipids and smooth muscle cells at its core (Libby et al., 2002). The proliferation of smooth muscle cells leads to a further thickening of the arterial wall and subsequent loss of lumen diameter. At this stage, the condition can progress to form a plaque that occludes the vessel lumen or to the development a coronary thrombosis, both of which can result in a cardiovascular event (Luis, 2000).

The etiology of atherosclerosis is complex and despite years of research, is not completely understood. Further, disease initiation and / or progression of lesions and their maturity are difficult to detect and track in humans (Luis, 2000). Consequently, risk factors and clinical events (i.e. heart attack or stroke) have traditionally been used to estimate disease risk and severity. Specifically, strategies for the prevention and management of atherosclerosis primarily target cholesterol, particularly fasting levels of low density lipoprotein (LDL) cholesterol, as a key indicator of CVD risk (Genest et al., 2003). While there is long standing evidence that LDL cholesterol is causally related to heart disease , research shows that many patients who present with a clinical event have normal fasting lipid concentrations (Cabezas and Erkelens, 2000; Mamo and Proctor, 2002).

Recently, post-prandial lipemia has emerged as a key contributor to CVD risk and its progression. For instance, increased plasma concentrations of intestinally-derived chylomicrons and their remnants have been identified in normolipidemic patients with CAD (Meyer et al., 1996; Rajanatman et al., 1999; Weintraub et al., 1999; Redgrave et

al., 2004). Studies also show that elevated plasma levels of chylomicrons are associated with chronic conditions that accelerate atherogenesis, such as familial hypercholesterolemia, insulin resistance, and type 2 diabetes mellitus (T2DM) (Tomkin and Owens, 2001; Lairon et al., 2007). Chylomicrons are associated with the initiation and progression of atherosclerosis, because remnant particles are small enough to enter the vessel wall and become entrapped in sub-endothelial space (see detailed description in Section 2.2.3, Figure 2-2) (Mamo and Wheeler, 1994; Mamo et al., 1998; Proctor et al., 2002). Furthermore, Proctor et al. (2004) have shown that chylomicrons can deliver more cholesterol to the vessel wall than LDL cholesterol on a per particle basis.

As the number of studies linking post-prandial metabolism and chronic disease increases, interest has grown in the use of chylomicron metabolism as a possible indicator of early CVD risk. This, in turn, has raised the question of what method might be most appropriate to accurately detect of chylomicrons in plasma. However, the direct determination of chylomicrons and their remnants in fasting and post-prandial samples has been hampered by the presence of hepatically-derived lipoproteins of similar size and composition (Jackson and Wiliams, 2004). Further, the detection of chylomicrons has proven difficult because they can be found at different stages of catabolism in plasma during the post-prandial phase, and therefore, are heterogeneous in size and composition (Cohn, 1999). To date, there are only a handful of techniques able to measure chylomicron concentrations. Traditionally, ultracentrifugation has been used to isolate a triglyceride-rich (TRL) lipoprotein fraction containing newly synthesized (nascent) intestinal chylomicrons at a density < 1.006 g/ml. More recently, an antiserum to apolipoprotein (apo) B48 has been raised as an exclusive marker of chylomicrons; this has allowed for the specific and direct determination of chylomicrons from whole plasma (e.g., immunoblotting and enzyme-linked immunosorbent assays). At present,

the concentration of apo B48 is neither easily determined nor standardized. Consequently, it is not surprising that apo B48 concentrations in the literature vary as much as 10-100 fold thereby confounding comparisons between studies (Jackson and Williams, 2004). The method used in the detection of chylomicrons is relevant because some methods reflect the plasma concentration of larger, nascent chylomicrons (e.g., TRL fractions) whereas others represent smaller remnant particles (e.g., measurement from whole plasma) (Cohn, 1999). In order to provide the most accurate interpretation of post-prandial lipemia, it is important to employ a method that can detect small chylomicron remnants, because the size of the particle is important to its' potential atherogenicity. Indeed, there is evidence that chylomicron particles found in density fractions > 1.006 g/ml are potentially more atherogenic and may be a better predictor of disease risk than the measurement of chylomicrons from TRL fractions (Mamo et al., 1998; Cohn, 1999). Thus, the specific and accurate detection of chylomicrons from whole plasma may potentially capture remnant particles that are of interest.

To our knowledge, no published reports have examined the methodological differences between the use of whole plasma or density fraction < 1.006 g/ml to detect apo B48 among individuals who are at greater risk from atherogenic effects compared to a healthy population.

1.2 Thesis Aim

The primary aim of this thesis was to contribute knowledge on how best to measure chylomicron metabolism, either from whole plasma or from a triglyceride-rich lipopoprotein (TRL) fraction (density < 1.006 g/ml), in order to provide the most accurate interpretation of post-prandial lipemia.

To achieve this aim, we measured apo B48 in two different study populations: adults with hyper-insulinemia and type 1 diabetes mellitus (T1DM). In the first study (Chapter 3), we investigated methodological differences in a situation of established post-prandial lipemia (in this case, hyper-insulinemia), allowing our findings from apo B48 measurements in whole plasma and density fractions < 1.006 g/ml to be compared and validated with knowledge from current literature. Subsequently, we applied the most accurate measure of apo B48 (whole plasma or TRL fraction) to investigate post-prandial metabolism in individuals with T1DM (Chapter 4), as the relationship between apo B48 and atherogenesis has not been extensively explored in this population.

1.3 General Hypotheses

The hypotheses for this thesis are as follows:

1. The detection of fasting apolipoprotein B48 (apo B48) from whole plasma will be statistically greater than apo B48 from triglyceride-rich lipoprotein (TRL) fractions (density < 1.006 g/ml) in a condition of post-prandial lipemia.
2. Apo B48 associated-chylomicron concentrations during the post-prandial state, as determined by area under the curve (AUC), will be elevated in a condition of post-prandial lipemia when measured from whole plasma relative to a TRL fraction (density < 1.006 g/ml).
3. The measurement of apo B48 from whole plasma will detect a significantly greater proportion of chylomicron particles at fasting and post-prandial, as determined by AUC, in a sample of subjects with brittle type 1 DM compared to healthy controls.

1.4 Specific Objectives

To test the above hypotheses:

1. To determine apo B48 levels from both whole plasma and TRL fractions (d < 1.006 g/ml) during the fasting and post-prandial state, as determined by AUC, in a sample of hyper-insulinemic / insulin resistant subjects.
2. To examine chylomicron metabolism, as measured from whole plasma apo B48 concentrations at fasting and post-prandial AUC, in a free-living situation among normolipidemic subjects with type 1 DM compared to healthy controls.

Chapter 2: Literature Review

2.1 Lipoproteins

2.1.1 Introduction

Lipids play a variety of critical biological roles. They are the main storage form of energy in most organisms, as well as a major constituent of cell membranes (Nelson and Cox, 2000). Specialized lipids serve as pigments (retinal, carotene), detergents (bile salts), cofactors (vitamin D), hormones (vitamin D), transporters, anchors for membrane proteins, and extracellular and intracellular messengers (eicosanoids). The main types of lipids found in plasma are triglycerides (TG), phospholipids, unesterified free fatty acids, unesterified cholesterol, and cholesterol esters (Nelson and Cox, 2000). Lipids are essentially insoluble in water, and consequently, are carried from one tissue to another via macromolecules known as lipoproteins.

Lipoproteins are formed from specific carrier proteins, called apolipoproteins, combined with lipids to generate spherical complexes with hydrophobic lipids in the core and hydrophilic amino acid side chains at the surface (Nelson and Cox, 2000). Various combinations of lipids and proteins produce particles of different densities (Table 2-1). As the ratio of lipid to protein varies, the buoyancy and size of the lipoprotein particle changes accordingly. In general, as lipoproteins progress from low to high density, they display decreasing TG content and increasing cholesterol ester, phospholipid and protein. Thus, lipoproteins can be separated by size, density, and/or composition (Kritchevsky, 1986).

Table 2-1. Major Classes of Human Plasma Lipoproteins: Some Properties

Lipoprotein	Density (g/ml)	Size (nm)	Triglyceride (% lipid)	Cholesterol (% lipid)	Phospholipid (% lipid)
Nascent Chylomicron	< 1.006	100-400	80-95	2-7	3-9
VLDL	0.95-1.006	40-70	55-80	5-15	10-20
LDL	1.006-1.063	22.5-27.5	5-15	40-50	20-25
HDL	1.063-1.210	7.5-10	5-10	15-25	20-30

Modified from: Kritchevsky D. Atherosclerosis and nutrition. Nutr Int. 1986;2:290-297

2.1.2 Chylomicrons

Chylomicrons are intestinally-derived lipoprotein particles and transport dietary fatty acids and fat soluble vitamins to the tissues where they are consumed and / or stored as fuel. Chylomicrons can be recognized by the presence of their unique structural protein apolipoprotein (apo) B48 (Kane et al., 1980). Apo B48 is synthesized from the same gene as the hepatically-derived apo B100; however, a stop codon inserted half-way into intestinal mRNA accounts for a protein that is 48% the size of apo B100, and in humans, is exclusively secreted from the intestine (Kane et al., 1980). Apo B48 is ideal for the determination of chylomicron kinetics as it is indicative of particle number (only one apo B48 per chylomicron particle), essential for chylomicron assembly, and not transferred to other lipoproteins (Phillips et al., 1997).

Typically, nascent chylomicrons are TG-rich (88%), reflect dietary fatty acid intake, and carry 2-5% of the cholesterol pool (Olson, 1998). Due to the greater TG to protein ratio, nascent chylomicrons are large (100-400 nm) and buoyant. Therefore, chylomicrons are often termed 'triglyceride-rich lipoproteins' and are traditionally isolated at density < 1.006 g/ml, with a Svedberg flotation (Sf) value > 400.

However, the hydrolysis of chylomicrons generates a remnant that is considerably smaller than the nascent chylomicron, between 45 and 60 nm in diameter (Redgrave, 2004). As a result, chylomicron remnants are TG-depleted (70%), contain less phospholipid, and have a greater proportion of cholesterol ester (13%) relative to the nascent chylomicron (Cooper, 1997). Chylomicron remnants can exist in density fractions > 1.006 g/ml and can be found in the VLDL fraction (Cohn et al., 1999).

Due to their smaller size, chylomicron remnants are potentially atherogenic lipoprotein particles (Cohn et al., 1999; Tomkin and Owens, 2001). Fasting and post-prandial chylomicron concentrations, hypothesized to be mostly in remnant form, are elevated in a number of diet and lifestyle-related diseases and conditions such as obesity, insulin resistance, and type 2 DM (Chen et al., 1993; Curtin et al., 1996; Chan et al., 2002; Couillard et al., 2002). While chylomicron remnants exist in plasma at relatively low concentrations due to their rapid catabolism, these particles have been shown to deliver more cholesterol to the vessel wall than LDL cholesterol on a per particle basis (Proctor et al., 2004). Despite advancements in post-prandial research, chylomicron measurements have yet to be applied to a routine clinical setting.

2.1.3 Very Low Density Lipoproteins

In situations where the diet contains more fatty acid than is immediately required for fuel, they are converted into TG in the liver and packaged into very low density lipoproteins (VLDL) for transport to muscle and adipose tissue. VLDLs are secreted from the liver with apo B100 and apo E on their surface and acquire additional apolipoproteins (e.g. apo CI, II and III) in the blood compartment. VLDL particles consist of approximately 7-10% protein and 90-95% lipid, of which 50-55% is TG, 5-15% is cholesterol, and 10-20% is phospholipid (Olson, 1998). VLDL particles share a similar composition to that of

chylomicrons due to the large proportion of core lipid and surface TG, and are also termed 'triglyceride-rich' lipoproteins. Consequently, VLDLs are isolated at a density which overlaps that of chylomicrons (density 0.95-1.006 g/ml) and a Sf rate of 20-400. Thus, the presence of apo B100 on the VLDL particle is one of the only means to distinguish VLDL from chylomicrons.

Traditionally, VLDL particles are not believed to be significantly involved in the development of atherosclerosis (Nordestgaard, 1992). Although the catabolism of VLDL removes up to 75% of lipids and reduces its size, the influx of VLDL to arterial vessels is much less compared to other lipoproteins such as LDL (Nordestgaard, 1992).

2.1.4 Low Density Lipoprotein Cholesterol

The loss of TG from VLDL particles can lead to the formation of cholesterol-rich lipoproteins known as low density lipoproteins (LDL). LDL particles express only apo B100 on its surface and are comprised of approximately 22% protein and 78% lipid, of which 5-15% is TG, 40-50% is cholesterol, and 20-25% is phospholipid. As a major cholesterol-carrying lipoprotein in the blood, LDL transports 70% or more of the plasma cholesterol at any one time. LDL particles have a density ranging from 1.006-1.063 g/ml, which is greater than that of chylomicrons and VLDL, despite its small size (22.5-27.5 nm) (Tomkin and Owens, 2001).

Based on long-standing evidence from experimental animal models, epidemiology, genetic forms of hypercholesterolemia, and clinical interventions, fasting plasma concentrations of LDL cholesterol have been identified as the primary target for reducing the risk of CVD (Genest et al., 2003). It is believed that increased arterial exposure to elevated concentrations of LDL is an atherogenic characteristic because LDL particles are small enough to enter subendothelial space and undergo modification (Tabas et al.,

2007). These modified forms of LDL are thought to be responsible for foam cell formation, a hallmark feature of early atherosclerotic lesions (Steinberg et al., 1989). Despite the substantial body of evidence supporting a positive relationship between LDL cholesterol and increased CVD risk, LDL cholesterol alone does not unequivocally explain the presence of cholesterol in atherosclerotic plaques. While LDL cholesterol is often raised in patients with coronary artery disease (CAD), approximately 40% have normal LDL levels (Cabezas and Erkelens, 2000; Mamo and Proctor, 2002).

2.1.5 High Density Lipoprotein Cholesterol

High density lipoprotein (HDL) particles begin in the liver and small intestine as small, protein-rich lipoproteins that contain relatively little cholesterol and no cholesterol esters. High density lipoproteins contain apo A-I, apo CI, apo CII, apo CIII, apo E, and the enzyme lecithin cholesterol acyl transferase (LCAT) which catalyzes the formation of cholesterol ester from lecithin and cholesterol. LCAT resides on the surface of nascent HDL and converts cholesterol and phophatidyl choline of LDL and VLDL to cholesterol esters. The cholesterol esters form the core of the disk-shaped nascent HDL, transforming it to a mature, spherical HDL particle. The cholesterol-rich, mature HDL subsequently returns to the liver where cholesterol is unloaded. Typically, a mature HDL particle consists of 5-10% TG, 15-25% cholesterol, and 20-30% phospholipid and is collected at density 1.063-1.21 g/ml.

In contrast to LDL, it is believed that HDL particles confer a protective effect as they function to remove excess cholesterol from blood and tissue, including the cholesterol loaded cells of atherosclerotic plaques, in reverse cholesterol transport pathways (Nelson and Cox, 2000). For instance, in one reverse transport pathway, apo AI in depleted HDL interacts with an active transporter, the ABC1 protein, in cholesterol-rich

cells. The apo A1 (and HDL particle) are taken up by endocytosis, then resecreted with a load of cholesterol to be transported back to the liver (Nelson and Cox, 2000). In a second pathway, the nascent HDL particle interacts with the plasma membrane receptor protein SR-B1 in cholesterol rich cells which triggers the passive movement of cholesterol from the cell surface into HDL (Nelson and Cox, 2000). Moreoever, epidemiological studies suggest that every 0.03 mmol/L (1 mg/dL) decrease in HDL cholesterol is associated with an increase of 2–3% in CVD (Wilson et al., 1988; Gordon et al., 1989). Accordingly, the Canadian Working Group on Hypercholesterolemia and Other Dyslipidemias has recommended the use of cholesterol:HDL cholesterol ratio as a secondary target of therapy (Genest, 2003)

2.2 Chylomicron Metabolism

2.2.1 Introduction

The post-prandial period results in a transient accumulation of lipoproteins that mediate the transport of dietary lipid in the circulation, namely chylomicrons as well as VLDL. The repeated flux of post-prandial lipoproteins through the intra-vascular space over the course of the day has led to the hypothesis that the metabolic response following the ingestion of a fat-containing meal could have pathological effects on the vessel wall (Tomkin and Owens, 2001; Redgrave, 2004). Significant advancements in post-prandial research have shown that chylomicrons are associated with atherosclerosis due to their ability to penetrate the vessel wall, be preferentially retained in subendothelial space, and induce macrophage lipid loading (Tomkin and Owens, 2001; Redgrave, 2004).

2.2.2 Chylomicrons and Cholesterol Metabolism

2.2.2.1 *Chylomicron Production and Secretion*

The production and secretion of chylomicron particles is dependent on the prandial state of the individual (Cartwright and Higgins, 1999). Even in the absence of dietary fat, nascent chylomicron particles are synthesized and secreted at a basal rate. However, the ingestion of a fat-containing meal leads to a stimulated increased production of chylomicrons from the intestine (Cartwright and Higgins, 1999). The formation of the chylomicron begins in the endoplasmic reticulum (ER) of the enterocyte and occurs in a two-step process (Mansbach and Gorelick, 2007). First, apo B48, apo A1V, cholesterol, and small amounts of TG are formed to produce a high-density chylomicron particle (Mansbach and Gorelick, 2007). The addition of large neutral lipids (TG and cholesterol) to this high-density chylomicron particle leads to the formation of a large, buoyant pre-

chylomicron (Mansbach and Gorelick, 2007). The TG core of the chylomicron reflects the dietary fatty acid composition of meals whereas the outer phospholipid layer of the particle reflects body fat (Luchoomun and Hussain, 1999). The pre-chylomicron buds from the ER surrounded by a membrane where it subsequently translocates to and fuses with the Golgi complex. At the Golgi, apo A1 attaches to the pre-chylomicron to form a mature particle which exits the Golgi complex in large transport vesicles and fuses with the basolateral membrane to be secreted into lymphatic collecting ducts (Mansbach and Gorelick, 2007).

2.2.2.2 *Chylomicron Clearance*

Once secreted, the first step in chylomicron clearance is the hydrolysis of the TG core by lipoprotein lipase (LPL), an enzyme on the surface of endothelial cells in skeletal muscle and adipose tissue that releases TG, free fatty acids and free cholesterol (Redgrave, 2004) (Figure 2-1). The actions of LPL are aided by the cofactor apo CII (Shelburn et al., 1980; Windler et al., 1980). Investigators have hypothesized that one of the functions of apo C is to ensure that chylomicrons have sufficient time to be hydrolysed in the plasma (Shelburn et al., 1980; Windler et al., 1980).

The hydrolysis of nascent chylomicrons leads to the formation of small, dense remnants. These remnant particles are removed from the circulation via receptor-mediated processes (Cooper et al., 1982). The composition of apolipoproteins associated with the chylomicron particle changes during the course of its metabolism; it gains more apo E while losing apo A. The addition of apo E to the chylomicron is of particular significance because apo E is the primary protein that interacts with the apo B100/apo E (or LDL) receptor for remnant clearance on the hepatocyte surface (Cooper et al., 1982). It has been shown that apo E has a high affinity for the apo-B100/apoE receptor, allowing for

the rapid clearance of chylomicron remnants from the circulation (Mahley and Innerarity, 1983).

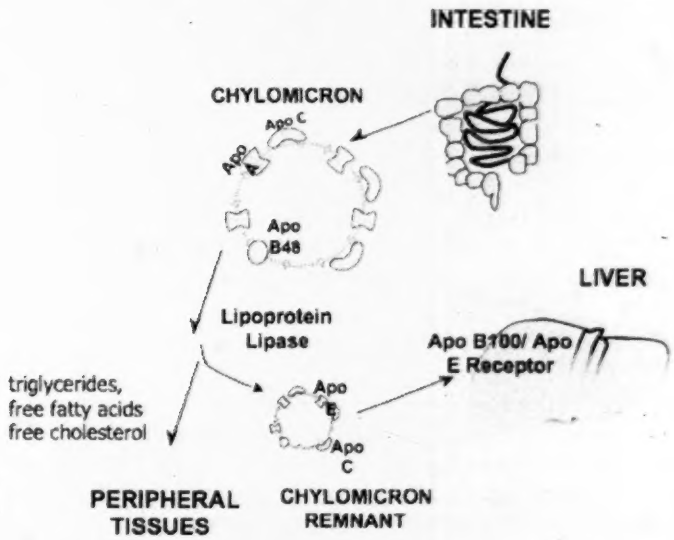

Figure 2-1. Schematic diagram of chylomicron hydrolysis and clearance by receptor-mediated processes

2.2.3 Chylomicrons in Atherogenesis

For individuals at risk for CVD, chylomicrons can be poorly hydrolysed and their clearance via hepatic receptor-mediated pathways can be delayed (Coppack, 1997; Malmström et al., 1997). There is also emerging evidence that both the production and secretion of chylomicron particles may be up-regulated during disease states with accelerated atherogenesis, including obesity, insulin resistance and type 2 diabetes mellitus (T2DM) (Haidari et al., 2007; Duez et al., 2006; Hogue et al., 2007). The etiological significance of increased levels of chylomicron remnants in the circulation is that these particles are small enough to enter and become entrapped within the intima of arterial vessels (Mamo et al., 1994; Mamo et al., 1998; Proctor et al., 2002) (Figure 2-2). Moreover, chylomicron remnants can be preferentially retained relative to other

lipoprotein fractions. In a study by Proctor et al. (2004), the delivery and efflux of both chylomicron remnants and LDL in the vessel wall were compared. While LDL particles had a higher rate of delivery, they effluxed more readily from arterial tissue compared to chylomicron remnants. Furthermore, chylomicron remnants can contribute substantially to the intimal deposition of cholesterol as they contain 42 times more cholesterol compared to LDL particles (Proctor et al., 2002). Hence, the accumulation of chylomicron remnants during conditions of post-prandial lipemia is relevant for CVD risk.

Upon entrapment in the subendothelial space, post-prandial lipoproteins are known to induce the inflammatory state by multiple mechanisms (Alipour et al., 2008). Chylomicrons have been found to have a chemotatic effect on human-derived monocytes *in vivo* (Proctor, 2000). Also, chylomicrons have been shown *in vitro* to induce the expression of endothelial cell adhesion molecule-1 (ECAM-1) and vascular adhesion molecule-1 (VCAM-1) on human endothelial cells (Moers et al., 1997). Monocyte recruitment and adhesion to the endothelium is an important step in the atherogenic process as monocytes differentiate to form macrophages at the site of the atherosclerotic lesion. Gianturco et al. (1998) have demonstrated that there are specific receptors on the macrophage for apo B48-containing lipoproteins. Moreover, Brown et al. (2000) found that lipoprotein uptake by the apo B48 receptor converts macrophages into lipid filled foam cells and advances the fatty streak.

As the atheroma matures, T-cells release hydrolytic enzymes such as metalloproteases which lead to local cell death (Ross, 1999). The accumulation of necrotic inflammatory cells, smooth muscle cells and fibrous tissues restructures the lesion and forms a fibrous cap (Ross, 1999). Injury to the artery wall may affect its ability to dilate and improve blood flow (Ross, 1999). Many processes are believed to affect the normal functioning of the vessel wall during the development of atherosclerosis. One possible mechanism is

the impairment of endothelium-dependent vasorelaxation by chylomicron remnants (Grieve et al., 1998; Doi et al., 1998). Thus, the inability of the artery to compensate in the presence of a lesion (i.e., by dilating) may lead to an occlusion in blood flow (Ross, 1999). Alternately, an eventual weakening or tearing of the plaque may cause hemorrhaging and luminal thrombosis. In either case, the occlusion of the vessel may result in ischemia, myocardial infarction and / or stroke (Schroder and Falk, 1996).

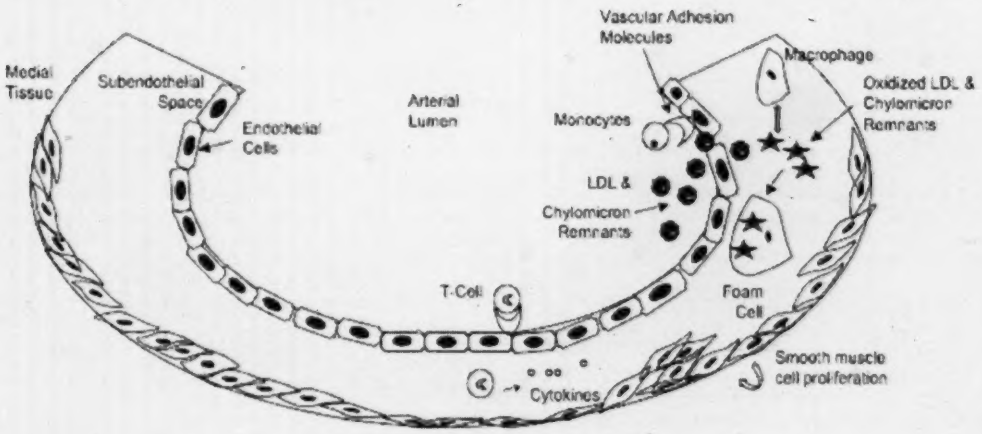

Figure 2-2. Schematic diagram of the initiation of atherosclerosis: lipid deposition and entrapment in the vessel wall

2.2.4 Chylomicrons and Clinical Studies of Coronary Artery Disease

Clinically, the persistent and substantial impairment of chylomicron remnant clearance and metabolism is pertinent for individuals with or at risk of CAD. This is particularly relevant for subjects whose concentrations of LDL cholesterol are within the normal range (Meyer et al., 1996; Rajanatman et al., 1999; Weintraub et al., 1999; Redgrave, 2004). In a study by Meyer et al. (1996), fasting apo B48 levels were significantly higher among normolipidemic subjects (n = 24; mean age: 60 ± 2 years) with CAD (as verified by coronary angiography) compared to healthy controls (0.25 ± 0.03 *versus* 0.15 ± 0.03 integrated optical density units; $p < 0.05$) Similarly, Rajanatman et al. (1999) found

increased fasting apo B48 levels in post-menopausal women (n = 24) with CAD versus women without CAD (3.10 ± 0.39 mg/L *versus* 1.67 ± 0.22 mg/L; $p < 0.001$) despite similar serum TG, total cholesterol and total apo B concentrations. In addition to elevated fasting apo B48, subjects with CAD demonstrated post-prandial lipemia compared to controls, indicated by the higher apo B48 incremental area under the curve (iAUC) (20.6 ± 2.8 *versus* 11.0 ± 1.2; $p<0.01$) (Rajanatman et al., 1999).

2.2.5 Conclusion

Increased post-prandial lipemia is recognized as a CAD risk factor, and impaired metabolism of chylomicrons and their remnants is believed to contribute directly to the initiation and progression of atherosclerosis (Tomkin and Owens, 2001). Although there is strong rationale for measuring chylomicrons to assess metabolic lipid abnormalities or risk of CAD, this procedure has not yet been applied to routine clinical practice. Currently, the expansion of the field of chylomicrons has been limited. One of the main reasons is that no agreement exists as to the best way to quantify chylomicrons (Cohn, 2006). Thus, a comparison of chylomicron methodology (whole plasma and TRL fractions) forms the basis for this thesis. A summary of the different approaches that have been undertaken to detect chylomicrons is discussed in section 2.4.

2.3 Chylomicron Metabolism in Insulin Resistance / Hyper-insulinemia and Type 1 Diabetes Mellitus

2.3.1 Chylomicron Metabolism in Insulin Resistance / Hyper-insulinemia

2.3.1.1 *Introduction*

With the prevalence of diabetes, particularly T2DM, reaching epidemic proportions in Canada and worldwide, achieving a better understanding of the role of insulin resistance in chronic disease risk is gaining prominence (Harris et al., 1998; Leiter et al., 2001; Dunstan et al., 2002). An elevated chylomicron response following a meal challenge has been demonstrated in insulin resistance (Abbasi et al., 1999; Ai et al., 2000; Harbis et al., 2001; Duez et al., 2006) as well as other conditions in which insulin resistance is a common clinical symptom, such as obesity, the metabolic syndrome, and T2DM (Curtin et al., 1996; Chen et al., 1999; Mekki et al., 1999; Chan et al., 2002). Therefore, in this thesis, we have selected hyper-insulinemia / insulin resistance as a model of post-prandial lipemia to better understand the clinical applicability of apo B48 methods (Chapter 3). As such, the following provides further background on chylomicron metabolism in relation to insulin resistance and diabetes.

2.3.1.2 *Physiological Role of Insulin*

Insulin is a peptide hormone secreted by the β cells of the pancreatic islets of Langerhans and plays a pivotal role in regulating cellular energy supply and directing anabolic processes of the fed state (Burks and White, 2001). Insulin is essential for the intra-cellular transport of glucose, via glucose transport protein 4, into insulin-dependent tissues such as muscle and adipose tissue (Burks and White, 2001). By signaling an abundance of exogenous energy following the ingestion of a meal, insulin suppresses adipose tissue fat breakdown and promotes adipose tissue synthesis (Karam, 1997). In

muscle, glucose entry into cells enables glycogen to be synthesized and stored and for carbohydrates, rather than fatty acids or amino acids, to be utilized as the immediately available energy source for muscle contraction (Karam, 1997). Therefore, insulin promotes glycogen and lipid synthesis in muscle cells while suppressing lipolysis and gluconeogenesis from muscle amino acids.

2.3.1.3 *Chylomicrons and Clinical Studies of Insulin Resistance / Hyper-Insulinemia*

Insulin resistance is a condition in which the adipose, skeletal muscle, and hepatic cells of the body become resistant to the effects of insulin; the normal response to a given amount of insulin is reduced (Reaven, 2004). Consequently, insulin secretion from pancreatic β cells is increased in an attempt to maintain normal blood glucose levels in a process known as compensatory hyper-insulinemia (Wilcox, 2005). Impairment of insulin secretion and defects in insulin action frequently coexist in the same individual, and it is often unclear which abnormality, either together or alone, is the primary cause of the hyperglycemia. Further, insulin resistance precedes the development of type 2 DM (Wilcox, 2000). A variety of approaches have been utilized to assess insulin resistance (Wilcox, 2005). The most robust methods for measuring insulin resistance are limited to research settings due to their high cost, invasiveness and need for clinical skill (e.g., euglycaemic hyper-insulinemic clamp, frequently sampled intravenous glucose tolerance test) (Wilcox, 2005). Fasting plasma insulin is the most widely used clinical indicator of insulin resistance (Wilcox, 2005).

Importantly, insulin resistance / compensatory hyper-insulinemia increases the risk of CVD in non-diabetic individuals (Pyorala, 1979; Després et al., 1996; Yip et al., 1998; Zavaroni et al., 1999). However, the role of insulin resistance in CVD risk is complex due to the fact that multiple CVD risk factors exist in individuals with this condition (Wilcox,

2005). In this context, increasing attention has been given to the atherogenicity of intestinally-derived chylomicron particles. It is now appreciated that individuals with insulin resistance / hyper-insulinemia have elevated concentrations of post-prandial lipoproteins (Abbasi et al., 1999; Ai et al., 2000; Harbis et al., 2001; Duez et al., 2006). For instance, Harbis et al. (2001) induced acute hyper-insulinemia among 10 healthy men (n = 10; 21-30 years of age) by two methodological approaches. First, various isolipidic test meals were used to stimulate different degrees of hyper-insulinemia. Apo B48 plasma concentration and insulin plasma concentration were found to be strongly correlated (r^2 = 0.70; p = 0.0001). Second, by utilizing a 3-hour hyperinsulinemic-euglycemic clamp, portal hyper-insulinism delayed and exacerbated the accumulation of apo B48 chylomicron-containing particles. Collectively, Harbis et al. (2001) found that increased chylomicron concentrations were associated with hyper-insulinemia in the absence of insulin resistance. Furthermore, Duez et al (2006) examined apo B48 metabolism in the fed state among 14 hyper-insulinemic, insulin resistant men (31-60 years of age) with a 15-hour primed constant infusion of [D3]-L-leucine. In this study, apo B48 intestinal production rate was significantly higher in hyper-insulinemic, insulin resistant subjects (1.73 ± 0.39 *versus* 0.88 ± 0.13 mg/kg/day; p < 0.05) and correlated with fasting plasma insulin concentrations (r = 0.558; p = 0.038). The relationship between insulin resistance and post-prandial chylomicrons has been frequently explored in the literature. Therefore, an insulin resistant / hyper-insulinemic population was utilized in this thesis to compare apo B48 measurements from whole plasma and density fractions < 1.006 g/ml.

2.3.1.4 *Chylomicrons and Clinical Studies of Overweight / Obesity*

Insulin resistance increases with greater body mass index (BMI) (weight in kilograms divided by height in meters squared), waist circumference and waist-to-hip ratio (WHR) (Aronne and Segal, 2002). Thus, it is not surprising that a positive relationship between obesity and post-prandial lipemia has also been repeatedly reported in the adult population (Mekki et al., 1999; Watts et al., 2001; Chan et al., 2002; Couillard et al., 2002). Couillard et al. (2002) found in a sample of 43 men (mean age 43.1 ± 9.6 years) that visceral abdominal fat was significantly associated with a decreased catabolism and an increased accumulation of apo B48-containing chylomicron particles ($r = 0.30$-0.44; $p < 0.05$). Similarly, Mekki et al. (1999) found elevated concentrations of chylomicron particles in abdominally obese women (WHR > 0.80 cm) compared to controls who were either lean or gynoid obese ($n = 24$, age 24-57 years; $p < 0.05$ for both comparisons). More recently, evidence has emerged suggesting that overweight children ($n = 40$; 7-11 years old) also have chylomicron concentrations equivalent to that found in adults diagnosed with CVD (Nzekwu et al., 2007).

2.3.1.5 *Chylomicrons and Clinical Studies of the Metabolic Syndrome*

Insulin resistance and obesity often exist among a constellation of other symptoms and conditions which are risk factors for chronic diseases. Many obese individuals have the metabolic syndrome (Lairon et al., 2007). However, there is no consensus regarding the operational definition of the metabolic syndrome. In 1998, the World Health Organization proposed a definition of the metabolic syndrome as 3 criteria among the following: elevated waist circumference (> 102 cm in men and > 88 cm in women); elevated triglyceridemia (≥ 1.7 mmol/l); low HDL cholesterol (< 1.0 mmol/l in men and < 1.3 mmol/l in women); elevated glycaemia (≥ 6 mmol/l); hypertension (systolic blood

pressure ≥ 130; diastolic blood pressure ≥ 85 mm Hg) (Alberti and Zimmet, 1998). More recently, the United States Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (2001) provided a definition that does not include a measure of insulin resistance and is based on the following 3 or more criteria: elevated waist circumference (> 102 cm in men and > 88 cm in women); TG ≥ 1.7 mmol/l; fasting plasma glucose ≥ 6.0 mmol/l; low HDL cholesterol (< 1.0 mmol/l in men and < 1.3 mmol/l in women); blood pressure ≥ 130/85 mm Hg. While post-prandial dyslipidemia is a common feature among those at increased metabolic risk (Mekki et al., 1999; Kim et al., 2002; Lovegrove et al., 2002; Harbis et al., 2004), the complexity of this condition means that subject selection is more difficult due to their metabolic heterogeneity.

2.3.1.6 *Chylomicrons and Clinical Studies of Type 2 Diabetes Mellitus*

The progressive inability of pancreatic β cells to compensate for chronic insulin resistance with sufficient hyper-insulinemia signals the clinical onset of T2DM (Reaven, 2004). T2DM is the most common form of diabetes, accounting for approximately 90-95% of all cases (Canadian Diabetes Association, 2003). T2DM ranges from a predominantly insulin resistant condition with relative insulin deficiency to a predominantly insulin secretory defect with insulin resistance (Canadian Diabetes Association, 2003). The diagnostic criteria for T2DM and the plasma glucose thresholds are as follows: fasting plasma glucose ≥ 7.0 mmol/L or casual (any time of day) plasma glucose ≥ 11.1 mmol/L plus symptoms of diabetes (polyuria, polydipisia, unexplained weight loss, etc), or 2 hour plasma glucose ≥ 11.1 mmol/L following a 75-g oral glucose tolerance test (Canadian Diabetes Association, 2003; World Health Organization, 2006).

It is well established that the post-prandial profile is elevated in people with T2DM compared to healthy controls (Curtin et al., 1996, Mero et al., 2000; Taniguchi et al.,

2000; Ai et al., 2001; Dane-Stewart et al., 2003). Curtin et al. (1996) found that the post-prandial apo B48-associated chylomicron concentration was greater among subjects with T2DM ($n = 18$; age 62 ± 0.3 years). Moreover, participants with T2DM exhibited both a prolonged increase and a later post-prandial peak of chylomicron particles compared to healthy controls ($p < 0.01$). Because individuals with T2DM have an increased risk of developing atherosclerosis, researchers have explored the post-prandial response of subjects with T2DM and CAD. For instance, Mero et al. (2000) conducted a case-control study of 43 T2DM subjects with severe ($n = 26$) and mild ($n = 16$) CAD (verified by coronary angiogram). In this study, post-prandial apo B48 concentrations were elevated in participants with T2DM, even in those with only mild CAD ($p < 0.003$). Furthermore, the most significant coronary stenosis correlated with chylomicron apo B48 area under the curve (AUC) ($r = 0.548$; $p = 0.01$). Most nutritional studies performed in the field of post-prandial metabolism utilize subjects with T2DM (Lairon, 2007). However, many of these participants are also overweight and / or exhibit features of the metabolic syndrome, making subject selection of particular importance in order to minimize intra-sample heterogeneity.

2.3.1.7 *Mechanisms Underlying Abnormal Chylomicron Metabolism in Insulin Resistance / Hyper-Insulinemia*

Individuals with insulin resistance have an exaggerated and prolonged chylomicron response in the blood following a dietary fat challenge (Howard, 1999). The mechanisms underlying the overaccumulation of chylomicron particles have not been fully characterized and studies have suggested a mixed contribution of impaired LPL activity (Howard, 1999), reduced recognition of hepatic receptors (Coppack et al., 1997; Malström et al., 1997), and intestinal overproduction (Haidari et al., 2002; Lewis et al., 2005) (Figure 2-3).

Post-prandial chylomicron overaccumulation may indirectly result from increased hepatic VLDL production during the insulin resistant state. Hepatic insulin resistance leads to the inappropriate secretion of VLDL in the fasting and post-prandial phase (Malström et al., 1997) because poor insulinization increases adipocyte lipolysis and provides free fatty acid (FFA) substrate for hepatic VLDL production (Chen et al., 1987; Swislocki et al., 1987). Since VLDL and chylomicron particles can compete for LPL activity, an increase in hepatic VLDL release may prolong the residence time of chylomicrons in circulation (Havel, 1994).

Elevated post-prandial chylomicron concentrations in insulin resistance may result directly from decreased LPL activity. It has been hypothesized that alterations in biologically active LPL functioning may arise from defects in cellular production (Semenkovich et al., 1989; Tavangar et al., 1992) and / or transport of LPL to the luminal side of capillary endothelial cells (Knutson, 2000). There is also evidence to suggest that insulin therapy improves LPL activity in subjects with insulin resistance and T2DM (Taskinen and Nikkila, 1979; Yost et al., 1995; Bagdade et al., 1997). Furthermore, the chronic hyperglycemia associated with insulin resistance can lead to the glycation of proteins such as apo E. Changes in ligand properties have been shown to impair the normal interaction of the apo B100 / apo E receptor with chylomicron remnant particles, and thereby affecting remnant clearance (Curtiss and Witztum, 1985; Mamo et al., 1990). Finally, emerging evidence indicates that both the production and secretion of chylomicron particles is up-regulated during disease states such as insulin resistance and T2DM (Adeli and Lewis, 2008). Recent studies utilising cultured primary enterocytes from fructose-fed, insulin-resistant hamsters have found an increased secretion of intestinally-derived chylomicron particles (Haidari et al., 2002; Lewis et al., 2005). The findings from animal studies have been confirmed in humans using a primed constant

infusion of L-[5,5,5-D_3] leucine in the fed steady state (Duez et al., 2006; Hogue et al., 2007). A greater intestinal secretion of chylomicron particles has been demonstrated in subjects with hyperinsulinemia (Duez et al., 2006) and T2DM (Hogue et al., 2007) compared to healthy controls.

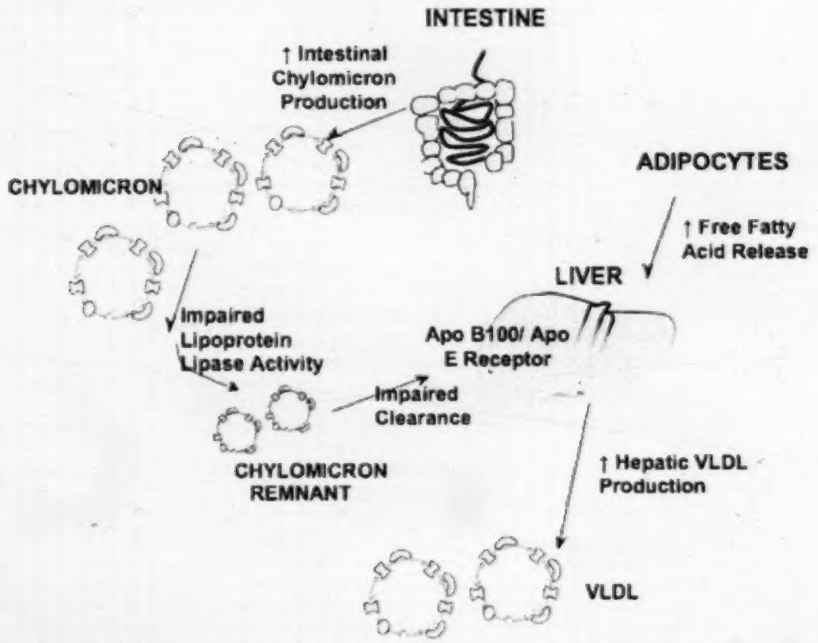

Figure 2-3. Schematic diagram of aberrant chylomicron metabolism in insulin resistance / hyper-insulinemia

2.3.2 Chylomicron Metabolism in Type 1 Diabetes Mellitus

2.3.2.1 *Introduction*

Type 1 diabetes mellitus is marked by cellular-mediated autoimmune destruction of pancreatic β cells resulting in an absolute deficiency of insulin secretion (Canadian Diabetes Association, 2003). Markers of immune destruction of β cells include auto-antibodies to insulin, auto-antibodies to glutamic acid decarboxylase, and auto-antibodies to tyrosine phosphatases IA-2. Usually, one or more these auto-antibodies are present in 85–90% of individuals when fasting hyperglycemia is initially detected (Canadian Diabetes Association, 2003). The plasma glucose threshold for a clinical diagnosis of T1DM is the same as T2DM (section 2.3.1.6).

As with insulin resistance and T2DM, individuals with T1DM are at an increased risk (3-4 fold) of developing CVD compared to an age-matched non-diabetic population (Kannel and McGee, 1979). However, despite the increased CVD risk, classic fasting lipid indices (HDL cholesterol, LDL cholesterol and / or TG concentrations) are often normal in individuals with T1DM (Howard, 1987). Moreover, the increased atherosclerotic risk may not be attributed to other non-lipid risk factors, as there is no increased prevalence of hypertension, obesity, or smoking in this population (Howard, 1987). Currently, the etiology for increased atherosclerotic risk in T1DM is not well understood and shapes the foundation of Chapter 4 in this thesis.

2.3.2.2 *Chylomicrons and Studies in Type 1 Diabetes Mellitus*

While the association of impaired chylomicron metabolism appears better defined in dietary and lifestyle related diseases (obesity, insulin resistance, metabolic syndrome and T2DM), the role of these particles in T1DM is unclear. In T1DM, a limited number of animal studies have examined chylomicron metabolism in streptozotocin-induced

diabetic rats (Redgrave and Snibson, 1977; Levy et al., 1985). In an autologous study, Redgrave and Snibson (1977) injected labeled chylomicrons from thoracic duct lymph in control and diabetic rats following a test meal containing ^{14}C cholesterol and 2-^{3}H glycerol trioleate to determine if chylomicron clearance rates differed between the 2 groups. Redgrave and Snibson (1997) found that diabetic rats demonstrated a decreased clearance of chylomicron particles compared to controls. Furthermore, a human study of men with T1DM (n = 6; mean age 33 ± 4.9 years) found an elevation of apo B in the TRL fraction (Sf 100-400) compared to healthy controls (Georgopoulos and Phair, 1991). To date, no studies have examined apo B48 remnant particle metabolism in the human T1DM population.

2.3.2 Conclusion

Conducting the two studies outlined in this thesis was important for several reasons. In relation to insulin resistance, evidence suggests that this condition precedes type 2 DM and places individuals at increased risk for CVD morbidity and mortality (Pyorala, 1979; Després et al., 1996; Yip et al., 1998; Zavaroni et al., 1999; Canadian Diabetes Association, 2003). Further, post-prandial lipemia is well established in this population (Howard, 1999). This allows for apo B48 measurements from whole plasma and density fractions < 1.006 g/ml to be compared and validated with knowledge from current literature. Finally, the method that most accurately measures chylomicron remnants in density fractions > 1.006 g/ml will be used to explore apo B48 metabolism in type 1 DM.

2.4 Detection of Chylomicrons

2.4.1 Introduction

As impaired chylomicron metabolism becomes increasingly recognized as a risk factor for CVD, it is only logical that researchers and clinicians suggest the need to measure plasma lipoprotein levels in the fed state. To date, there are only a few techniques available for the detection of chylomicrons, and these methods differ in their ability to detect small, potentially atherogenic remnant particles (Cohn, 2006). Thus, the final phase of this literature review provides an overview of methods that have historically been used to quantify chylomicron concentrations as well as newer techniques available.

2.4.2 Triglyceride-Rich Lipoprotein Fractions

The primary change elicited in the circulation following a fatty meal is a transient elevation in triglyceride-rich lipoproteins (TRL) derived from the intestine (chylomicron) and the liver (VLDL). Density grading and sequential ultracentifugation have traditionally been used to separate chylomicrons from VLDL because the density of buoyant, nascent chylomicron particles is slightly lower than that of VLDL (Cohn, 2006). Classically, chylomicrons have been collected from plasma by ultracentrifugation at density < 1.006 g/ml.

While sub-fractioning is common practice for the separation of lipoproteins, it is limited by variations in lipoprotein particle size, particularly in the post-prandial state (Cohn et al., 1999). The size of the chylomicron is dependent upon the degree of delipidation that has occurred in the circulation prior to sampling. Thus, up to 50% of the total apo B48 pool can consist of smaller remnants that are found in VLDL, and to a lesser degree, the LDL fraction (Cohn et al., 1999). As the size and density of the particle are important to

its' potential atherogenicity, the determination of chylomicrons with TRL sub-fractions may omit remnant particles at density > 1.006 g/ml that are of relevance to the etiology of atherosclerosis. Furthermore, ultracentrifugation remains a labor intensive process. Hence, researchers have often utilized proxy measures for the assessment chylomicrons and their remnants.

2.3.2 Indirect Measures

2.3.2.1 *Remnant-Like Particle Cholesterol*

In recent years, remnant-like particle cholesterol (RLP-C) concentrations have been used as an alternative approach to assess CVD risk associated with dysfunctional postprandial metabolism (Havel et al., 2000). RLP-Cs are partially catabolised lipoproteins derived from the lipolysis of intestinal apo B48-containing chylomicrons and / or hepatic apo B 100-containing VLDL. Thus, these small, dense remnants are TG-depleted as well as cholesterol and apo E enriched (Cohn et al., 1999).

It has been shown that RLP-C concentrations are elevated in obesity (Chan et al., 2002), insulin resistance (Abbasi et al., 1999; Ai et al., 2000; Ai et al., 2001; Ohnishi et al., 2002; Schaefer et al., 2002; Funada et al., 2004) and CAD (Higashi et al., 2001; Schaefer et al., 2001; Dane-Stewart et al., 2003; Imke et al., 2005). However, RLP-C measurements lack specificity and detect both apo B100 and apo B48-containing lipoproteins (Cohn, 2006). Thus, the use of RLP-C as a measure of post-prandial metabolism does not necessarily allow investigators to distinguish the separate actions of intestinally-derived chylomicron particles and hepatically-derived lipoproteins in the role of atherogenesis.

2.4.2.3 *Retinal Esters*

The measurement of post-prandial retinyl esters (RE) in the blood has been used to identify the presence of chylomicrons and their remnants (Cohn, 2006). The concept for the use of RE as a measure of the clearance of chylomicron particles is as follows: dietary lipophylic vitamin A molecules are esterified in the intestine, incorporated into the core of a chylomicron particle, and remain associated with the chylomicron from intestinal secretion until hepatic remnant clearance. A decreased clearance of plasma RE after vitamin A ingestion has been demonstrated in studies with T2DM subjects (Chen et al., 1993) and CAD (Simpson et al., 1990; Groot et al., 1991; Weintraub et al., 1996). Although the measurement of REs has been widely used to assess post-prandial metabolism, several studies have drawn attention to the shortcomings of this approach (Cohn et al., 2006). For example, Rajaratnam et al. (1999) studied 54 women (mean age: 52.3 ± 0.5 years) and found that the total post-prandial AUC and peak iAUC for RE did not differ between women with CAD (defined as angiographically proven stenosis of 50% in at last one coronary artery) *versus* matched controls. However, in the same study, determination of chylomicron concentrations with other methods (sodium dodecyl sulphate polyacrylamide gel electrophoresis [SDS-PAGE] and coomassie staining), showed that women with CAD had a significantly greater chylomicron AUC ($p < 0.001$) compared to controls and adjustments for fasting concentrations did not alter the post-prandial findings ($p = 0.01$). Similarly, a study by Mero et al. (2000) compared the post-prandial response of men and women with T2DM ($n = 43$; mean age: 57.7 ± 1.1 years) and CAD (defined as angiographically proven stenosis) to controls without CAD. While the measurement of RE did not find an elevated response in either plasma or chylomicron fractions (Sf > 400) among T2DM subjects with CAD *versus* controls, determination with SDS-PAGE and coomassie blue staining demonstrated a correlation

between chylomicron AUC and the most severe coronary stenosis (r = 0.461; p = 0.041). These differences may be attributed to the shortcomings of utilising RE as a proxy measure of chylomicron concentrations. Some studies have shown that more RE molecules are incorporated into larger chylomicron particles (Karpe et al., 1995). Furthermore, other investigators have reported a transfer of RE between lipoproteins, and consequently, RE molecules have been found in lipoprotein particles of hepatic origin (Krazinski et al., 1990; Cohn et al., 1993;). Thus, plasma concentration of RE may potentially be an unreliable marker of intestinally-derived chylomicron particles, thereby limiting its use as a method for assessing post-prandial lipemia.

2.4.2.4 *^{13}C Breath Test*

The *^{13}C* breath test is a functional assessment of chylomicron remnant metabolism and has been used to examine post-prandial metabolism in hyperlipidemic, obese and insulin resistant subjects (Redgrave et al., 2001; Watts et al., 2001; Chan et al., 2002; Dane-Stewart et al., 2003) (Table 2-3). The appearance of $^{13}CO_3$ in the breath is measured following the intravenous injection of a lipid emulsion labelled with cholesteryl ^{13}C-oleate (Redgrave et al., 2001). The lipid emulsion mimics the composition and physiology of chylomicron remnants, and their removal by receptor-mediated processes releases labelled ^{13}C that in turn is expired in the form of $^{13}CO_2$ (Redgrave et al., 2001).

Utilising the ^{13}C breath test in a cross-sectional study, Watts et al. (2001) found that centrally obese men (n=24; mean age: 43.4 ± 2.4) demonstrated a decreased fractional catabolic rate (FCR) of remnant-like particles compared with lean subjects (0.061 ± 0.014 *versus* 0.201±0.048 pools/h; p = 0.016). Similarly, Dane-Stewart et al. (2001) showed that post-menopausal women with type 2 DM (n=24; mean age: 60 ± 1 years) had an increased initial appearance of $^{13}CO_2$ over time *versus* non diabetic women (p <

0.01). Diabetic women also had a significantly lower FCR than non-diabetic women (0.06 ± 0.05 pools/hr *versus* 0.12±0.02 pools/hr; p= 0.007) (Dane-Stewart et al. 2001). Nevertheless, the breath test has not yet been used to identify individuals with CAD in the absence of other conventional risk factors. Furthermore, no commercial kits are yet available for the breath test.

2.4.4 Direct Measures of Chylomicron Metabolism

2.4.4.1 *SDS-PAGE*

Sodium dodecyl sulphate polyacrylamide gel electrophoresis is the most widely used approach for the detection of apo B48 containing lipoproteins and is the only means of distinguishing apo B48-containing chylomicrons from apo B100-containing VLDL particles (Jackson and Williams, 2004). Samples are first prepared through ultracentrifugation to create a TRL fraction (density < 1.006 g/ml) that is rich in newly synthesized chylomicrons (Jackson and Williams, 2004). Apo B48 proteins are then separated on a polyacrylamide gel based on molecular weight (260 kDa), and the proteins are visualized with coomassie blue staining (Jackson and Williams, 2004). The quantification of the apo B48 band is conducted by densitometric scanning of gels as there is a linear (Karpe and Hamsten, 1994) or power relationship (Kotite et al., 1995) between protein content and dye uptake depending on the type of stain used. Within the literature, apo B48 has been measured: (1) as a percentage of fasting or peak concentrations, (2) as a ratio to apo B100, (3) as absolute apo B48 concentrations from a standard curving using apo B100 derived from LDL or (4) with affinity purified apo B48 (Jackson and Williams, 2004).

While SDS-PAGE with coomassie staining is an established method for measuring apo B-48 concentrations in TRL fractions, it is a multi-step process with no universally

consistent protocol. Variability is introduced into this method due to the type of gel (rod *versus* slab), gel stain, and two-dimensional scanning methods used (Jackson and Williams, 2006). Thus, large ranges in fasting levels of apo B48 are observed from this method. Table 2-4 summarizes the studies that have employed ultracentrifugation, SDS-PAGE, and coomassie staining to determine chylomicron metabolism in subjects with obesity, insulin resistance, and CVD.

2.4.3.2 *Enzyme Linked Immunosorbent Assays*

Recently, the raising of an anti-serum to apo B48 has allowed for the direct determination of chylomicrons by enzyme-linked immunosorbent assay (ELISA). With the availability of an anti-serum to apo B48, Uchida et al. (1998), Lorec et al. (2000) and Sakai et al. (2003) have developed ELISAs for the specific determination of apo B48 directly from whole plasma without the need for isolating TRL fractions (Table 2-5). The procedure, originally designed by Lorec et al. (2000), utilises a competitive ELISA in which apo B48 anti-serum is coated on an ELISA plate and samples are pre-incubated with apo B48 anti-serum before addition to the plate. The amount of anti-serum bound on the plate is inversely proportional to the concentration of apo B48 in the samples. Uchida et al. (1998) and Sakai et al. (2003) have each developed sandwich ELISAs whereby monoclonal anti-apo B48 antiserum is coated onto the ELISA plate before incubation with serum samples. The concentration of apo B48 in samples is proportional to the amount of apo B48 bound to the monoclonal anti-serum. Lovegrove et al (1996) has employed human lymph as a standard for apo B48 in an attempt to mimic chylomicrons found in density fractions < 1.006 g/ml. However, Sakai et al (2003) has used recombinant human apo B48 containing lipoproteins derived from cell culture medium of rat hepatoma McA-RH7777 cell lines.

Although the ELISA method is specific and allows for a high throughput of samples, several methodological limitations remain. The storage and preparation of samples for the ELISA technique can affect the exposure of immunogenic sites on apo B48 (Jackson et al 2004). Further, the use of monoclonal antibodies in ELISA has made this approach less sensitive, due to fewer epitope binding sites, compared to immunoblotting with polyclonal antibodies. As the ELISA method is not yet standardized, a wide range of results are obtained in the literature which limits its clinical utilization and applicability (Jackson and Williams, 2004).

2.4.3.3 *Immunoblotting*

The raising of polyclonal (Lorec et al., 2000) anti-serums to apo B48 has allowed for the development of assays, such as immunoblotting, which are more sensitive in the detection of apo B48 containing chylomicron particles. Smith et al. (1997) developed a method utilising SDS-PAGE, followed by immunoblotting with commercially available anti-apo B antiserum, and visualization of apo B48 bands with enhanced chemiluminescence to quantify chylomicron particles. This technique has been shown to be 8-10 times more sensitive than coomassie blue staining (Smith et al., 1997). Importantly, this has allowed for the measurement of apo B48 containing chylomicrons directly from whole plasma without the need for prior fractionation or additional preparative steps. Smith et al. (1997) standardized apo B48 concentrations against a purified apo B48 protein of known mass to reduce intra- and inter-assay variability, while other researchers have presented apo B48 concentrations as a percentage of the internal standard (Peel et al., 1993).

The increased precision of immunoblotting has resulted in studies that have consistently reported an elevation in apo B48 concentration from obese, insulin resistant, type 2

diabetic and dyslipidemic subjects compared to controls (Smith et al., 1997; Chan et al., 2002; Dane-Stewart et al., 2001; Redgrave et al., 2001; Dane-Stewart et al., 2003) (Table 2-6). While immunoblotting is a multi-step technique, it has been widely accepted as one of the most sensitive and accurate methods to quantify chylomicron concentrations from plasma (Jackson and Williams, 2004). Thus, immunoblotting was chosen for this thesis as the method that would best detect chylomicrons and their remnants directly from whole plasma.

2.4.5 Conclusion

Plasma lipoproteins of intestinal origin have been isolated and measured via a number of different biochemical procedures. While each method has contributed to our current understanding of post-prandial lipoproteins and their role in atherogenesis, they can not be considered equivalent. Whereas some methods reflect the plasma concentration of larger, TG-rich lipoproteins (e.g., ultracentrifugation and TRL fractions), others represent small, remnant particles (e.g., measurement from whole plasma with immunoblotting). It is critical that the methodology chosen to quantify chylomicrons is capable of detecting small remnant particles (in density fractions > 1.006 g/ml) in order to provide the most accurate interpretation of post-prandial lipemia and its contributions to CVD risk. Consequently, an appreciation of how best to measure chylomicron concentrations, either from whole plasma or TRL fractions, forms the premise for this thesis.

Table 2-2. Summary of Studies Reporting Chylomicron Concentrations from Retinyl Esters

Reference	Subjects	N (m/f)	Age (years)	Test Meal	Main Findings
Chen et al., 1993	T2DM	13/7	62 ± 2	Vitamin A: 60,000 U/m^2 body surface area with lunch	The post-prandial RE concentration was not different between those with and without T2DM in the chylomicron-containing Sf > 400 fraction.
Groot et al., 1991	CAD	40/0	51 ± 1	Oral fat load: 77.5 g fat, 500 mg cholesterol, 27,000 IU RE per U/m^2 body surface area	Subjects with and without CAD showed no differences in RE response in the ascending post-prandial phase (0-6 hours). CAD patients showed a marked delay in the clearance of RE (13.3 ± 1.6 mg/[Lxhr] *versus* 8.7 ± 1.1 mg/[Lxhr]; $p < 0.05$)
Sharrett et al., 1995	CAD	602	45-64	Oral fat load: 105 g fat, 300 mg cholesterol, 100 000 IU vitamin A U/m^2 body surface area	RE levels in white CAD subjects were similar to controls at fasting and 3.5 hrs, but RE levels were higher at 8 hrs ($p < 0.01$). Mean fasting and post-prandial RE levels were not different between black subjects with and without CAD.
Syvänne et al., 1994	T2DM and CAD	60/0	54 ± 1.6	Oral fat load: 78g fat, 490 mg cholesterol, 345,000 IU vitamin A	There were no differences in RE levels among diabetic subjects with or without CAD.

Table 2-2 continued. Summary of Studies Reporting Chylomicron Concentrations from Retinyl Esters

Reference	Subjects	N (m/f)	Age (years)	Test Meal	Main Findings
Meyer et al., 1996	CAD	0/24	60 ± 2	Oral fat load: 50 g fat per m^2 body surface, 60 000 IU RE per 50 g fat.	RE concentration at density < 1.006 g/ml was not different in CAD versus controls.
Weintraub et al., 1996	CAD	126/44	57 ± 1.4	Oral fat load: 50 g fat, 145 mg cholesterol, 60,000 IU RP per U/m^2 body surface area	The area below the RE curve (65.3 ± 28.3 *versus* 55.0 27.5 µmol/l·hr; p=0017) and non-chylomicron fraction RP curve (23.4 ± 15.0 *versus* 15.3 ± 8.9 µmol/l·hr; p < 0.001) was greater in CAD patients than controls.
Rajaratnam et al., 1999	CAD	0/54	52 ± 0.5	Oral fat load: 90 g fat, 432 mg cholesterol, 34,000 IU vitamin A	Total and peak incremental area under the curve (density < 1.006 g/ml) did not differ between subjects with CAD and controls.
Mero et al., 2000	T2DM & CAD	31/12	58 ± 1.1	Oral fat load: 100 000 IU vitamin A	RE response in plasma and chylomicron fractions (Sf >400) did not differ between diabetic subjects with and without CAD.
Mamo et al., 2001	Obese	35/0	46 ± 2.6	Oral fat load: fat content 47% wt/wt, 900 mg/kg RE	The area under the RE curve was significantly greater in obese subjects *versus* controls (3,120 ± 511 *versus* 1,308 ± 177 U/ml·hr; p = 0.03).

Table 2-3. Summary of Studies Reporting Chylomicron Concentrations from ^{13}C Breath Test

Reference	Subjects	N (m/f)	Age (years)	Test Meal	Main Findings
Redgrave et al., 2001	Hyper-lipidemia	32	28-65	Fasting	Compared with the normolipidemic men, the fractional catabolic rate of remnants was significantly decreased ($p < 0.006$) in subjects with type III dyslipidemia. In the group with type I hyperlipemia, the fractional catabolic rate was not different from the control group.
Watts et al., 2001	Obese	24/0	43 ± 2.4	Fasting	Centrally obese subjects had a decreased fractional catabolic rate of the remnant-like particles compared with lean subjects (obese: 0.06 ± 0.01 *versus* lean: 0.2 ± 0.05 pools/hr; $p = 0.016$).
Dane Stewart et al., 2003	T2DM	0/34	60 ± 1	Fasting	The initial appearance of $^{13}CO_2$ over time was greater in diabetic versus non diabetic women ($p < 0.01$). Diabetic women had a significantly lower fractional catabolic rate than non-diabetic women (diabetic: 0.06 ± 0.05 pools/hr *versus* control: 0.12 ± 0.02 pools/hr; $p= 0.007$)

Table 2-4. Summary of Studies Reporting Chylomicron Concentrations from Triglyceride-Rich Lipoprotein Fractions (d < 1.006 g/ml), SDS-PAGE & Coomassie Staining

Reference	Subjects	N (m/f)	Age (years)	Test Meal	Main Findings
Sharrett et al., 1995	CAD	602	45-64	Oral fat load: 105 g fat, 300 mg cholesterol	Apo B48 at fasting and 3.5 hours post-prandial did not differ.
Curtin et al., 1996	T2DM	8	62 ± 2.4	1340 kcal breakfast, 55% fat	Diabetic subjects had greater levels of apo B48 at fasting (2.2 ± 0.8 *versus* 0.5 ± 0.1 µg/ml; $p < 0.05$) and post-prandial ($p < 0.001$), with a prolonged increase and a later peak (6 hours: 7.7 ± 2.0 µg/ml *versus* 1.2 ± 0.2 µg/ml; $p < 0.01$).
Meyer et al., 1996	CAD	0/24	60 ± 2	Oral fat load: 50 g fat per m^2 body surface	Fasting apo B48 was higher in CAD cases (CAD: 0.25 ± 0.03 *versus* 0.15 ± 0.03 integrated optical density units; $p < 0.05$). A greater AUC response was also observed in CAD cases ($p = 0.01$).
Rajaratnam et al., 1999	T2DM and CAD	0/54	52 ± 0.5	Oral fat load: 90 g fat, 432 mg cholesterol	Women with CAD had a greater apo B48 post-prandial AUC ($p < 0.000$), particularly at 6 hours (CAD: 2.6 ± 0.6 mg/l *versus* control: 1.2 ±0.3 mg/l; $p < 0.01$). Adjustments for fasting concentrations did not change post-prandial findings ($p = 0.01$).

Table 2-4 continued. Summary of Studies Reporting Chylomicron Concentrations from Triglyceride-Rich Lipoprotein Fractions (d < 1.006 g/ml), SDS-PAGE & Coomassie Staining

Reference	Subjects	N (m/f)	Age (years)	Test Meal	Main Findings
Mero et al., 2000	T2DM and CAD	31/12	58 ± 1.1	Oral fat load: soybean oil 50 g/ m^2, glucose 50 g/ m^2, egg white protein 25 g/ m^2, dried egg yolk	Fasting and post-prandial apo B48 concentrations did not differ between diabetic subjects with and without CAD. The severity of the most significant coronary stenosis in angiography correlated with chylomicron apo B48 area under the curve ($r = 0.461$; $p = 0.041$).
Couillard et al., 2002	Obese	50/0	46 ± 9	Oral fat load: 60g fat/m^2 body surface area	Visceral abdominal fat was associated with an elevated postprandial apo B48 concentration ($r = 0.03$-0.44; $p < 0.05$). Total area under the curve: 5.8 ± 3.0 µmol·8hr/L; total area under the increment curve: 2.7 ± 1.8 µmol·8hr/L.
Duez et al., 2006	Hyper-insulinemic	14/0	49 ± 4.7	Fed steady-state: 18% fat, 20% protein, 62% carbohydrate	Apo-B48 intestinal production rate was increased in hyper-insulinemic subjects as measured by kinetic tracers ($p < 0.05$)
Hogue et al., 2007	Type 2 DM	24/0	54 ± 7.5	Fed steady-state: 40% fat	Diabetic subjects had higher apo B48 concentrations than controls (4.2 ± 2.0 mg/dL *versus* 0.9 ± 0.6 mg/dL; $p < 0.001$)

Table 2-5. Summary of Studies Reporting Chylomicron Concentrations from ELISA

Reference	Subjects	N (m/f)	Age (years)	Test Meal	Main Findings
Lovegrove et al., 2002	Obese and Insulin-resistant	0/24	62 ± 7	Stardardized breakfast (2469 kJ, 30 g fat) and lunch (3138 kJ, 44 g fat)	A significantly higher postprandial apo B48 response was observed in women with a higher BMI (>27 kg/m^2).
Sakai et al., 2003	Hyperlipidemia	588	37-74	Oral fat load: 30 g fat/m^2 body surface area	Serum apo B48 levels were 7-18 times greater in subjects with Type V (37.3 ± 14.9 µg/ml), and Type III (56.4 ± 7.9 µg/ml, n=5) hyperlipidemia compared to controls.
Valero et al., 2005	CAD	85/38	67 ± 13	Fasting	No differences in fasting apo B48 concentrations were found between those with CAD and controls (0.1 ± 0.1 *versus* 0.1 ± 0.1 µg/ml). There were also no differences between subjects with and without diabetes.

Table 2-6. Summary of Studies Reporting Chylomicron Concentrations from Immunoblotting

Reference	Subjects	N (m/f)	Age (years)	Test Meal	Main Findings
Chan et al., 2002	Obese	48/0	54 ± 9.0	Fasting	Obese subjects had significantly increased plasma concentrations of apo B48 (24.3 ± 8.8 *versus* 12.3 ± 2.8 mg/L; $p < 0.001$).
Dane-Stewart et al., 2001	Hyper-lipidemia	16/14	53 ± 2	Fasting	Subjects with familial dyslipidemia had elevated apo B48 concentrations *versus* controls (29.3 mg/L *versus*12.8 mg/L respectively; $p < 0.001$)
Mamo et al., 2001	Obese	35/0	46 ± 2.6	Oral fat load: fat content 47%	Compared with lean subjects, fasting concentrations of apo B48 was more than two-fold greater in obese individuals (obese: 31.5 ± 7.5 *versus* control: 12.7 ± 1.7 µg/ml; $p < 0.005$). AUC and incremental AUC were greater in obese subjects compared with lean controls (obese: 365 ± 71 *versus* control: 137 ± 18; $p < 0.005$, and obese: 9 ±17 *versus* control: 44 ± 12; $p= 0.03$, respectively).

Table 2-6 continued. Summary of Studies Reporting Chylomicron Concentrations from Immunoblotting

Reference	Subjects	N (m/f)	Age (years)	Test Meal	Main Findings
Redgrave et al., 2001	Hyperlipidemia	32	28-65	Fasting	Compared to normolipidemic participants, (6.9 ± 1.08 mg/L), subjects with type I hyperlipidemia (59.7 ± 9.9 mg/L) and type III hyperlipidemia (32.9 ± 4.5 mg/L had significantly elevated apo B48 concentrations ($p < 0.001$ and $p < 0.01$, respectively).
Dane-Stewart et al., 2003	T2DM	0/38	60 ± 1	Fasting	Women with T2DM had greater fasting apo B48 plasma concentrations (16.4 ± 1.2 *versus* 13.0 ± 0.9 mg/L; $p = 0.021$).

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Chapter 3: Quantification of Chylomicron Remnants in Fasting Hyper-Insulinemia

3.1 Introduction

Post-prandial dyslipidemia is a prominent feature of insulin resistance (Higashi et al., 2001; Ohnishi et al., 2002; Schaefer et al., 2002) and exists among a cluster of clinical symptoms in which individuals are predisposed to atherosclerosis (Zilversmit, 1995; Tomkin and Owens 2001; Proctor et al., 2002). The atherogenic nature of aberrant post-prandial metabolism has been attributed to the ability of cholesterol-dense chylomicron remnants to penetrate arterial tissue and become entrapped within arterial vessels (Mamo and Wheeler, 1994; Mamo et al., 1998; Proctor et al., 2000). Studies show that those with cardiovascular disease (CVD) may exhibit greater post-prandial concentrations of chylomicrons compared to healthy subjects, even when otherwise normolipidemic (Cohn et al., 1999). Thus, the measurement of post-prandial chylomicron metabolism has become of great interest as a potential index for the early and accurate detection of atherosclerosis and the insulin resistant state. However, the typical lipid profile used to assess CVD risk does not include a measure of chylomicron concentration.

Chylomicron metabolism is ideally assessed by monitoring the kinetics of apolipoprotein (apo) B48 in the post-prandial state. Apo B48 is structurally integral for the formation of chylomicrons, is endogenous, indicative of particle number, and not transferred to other plasma lipoproteins (Phillips et al., 1997). Metabolic studies of apo B48 have focused on the analysis of large triglyceride-rich lipoproteins (TRL) in the post-prandial state, a measurement which represents newly secreted apo B48 particles from the intestine. Ultracentrifugation techniques are often used to prepare TRL fractions at density < 1.006 g/ml, however, this method omits apo B48-associated particles found in remaining

fractions of whole plasma at density > 1.006 g/ml (Cohn et al., 1999; Sullivan et al., 2004). There is evidence that smaller apo B48-containing remnant particles located within density fractions > 1.006 g/ml are more atherogenic and may be more accurate predictors of disease risk (Mamo et al., 1998). Our laboratory has developed an SDS-PAGE and western immunoblotting method (Smith et al., 1997; Vine et al., 2007) such that apo B48 can be quantified accurately and directly from whole plasma without the need for prior fractionation or additional preparative steps (Vine et al., 2007). In the present study, plasma was collected in 12 men during a metabolically fed, steady-state which was achieved through the repeated intake of high fat liquid meals (Duez et al., 2008). Apo B48 concentration was determined in both whole plasma and corresponding TRL samples. The aim of this study was to examine if the preparation of the sample for measurement of apo B48 impacts the ability to identify dysfunctional post-prandial lipoprotein metabolism, specifically apo B48 remnant particles, in men with features of insulin resistance and fasting hyper-insulinemia.

3.2 Methods

3.2.1 Subjects

Subjects were normo-glycemic and normo-lipidemic men ages 30 to 57 years, with body mass index (BMI) values ranging from 22.8 kg/m^2 to 31.7 kg/m^2. Twelve men were selected from the saline group of a previously reported study (Duez et al., 2008). All participants had normal fasting lipid values with total plasma cholesterol < 5.6 mmol/L, low density lipoprotein (LDL) cholesterol < 4.1 mmol/L, high density (HDL) cholesterol > 0.9 mmol/L, triglycerides (TG) < 4.1 mmol/L, and fasting blood glucose < 6 mmol/L. All men had no prior history of cardiovascular, gastrointestinal, renal pulmonary disease or systemic illness, or surgical intervention within six months prior to the study.

All subjects gave their written consent to participate in the study, which was approved by the ethics board of the University Health Network, University of Toronto.

3.2.2 Achieving the Fed Steady State

Participants were fasted for 13 hours the evening prior to the study day and blood was drawn by an intravenous catheter inserted into their forearm vein. Subjects were provided with identical hourly volumes of a liquid food supplement, Hormel Great Shake Plus™ (Hormel Health Labs), for the first 3 hours, followed by the ingestion of the same formula every half hour for the remainder of the study (Duez et al., 2008). Blood was drawn at 0 hours (fasting) and during the fed steady-state at 6, 8, 12, 15, and 17 hours following the first meal and collected in a tube with 0.1% EDTA.

Each volume of the supplement was equivalent to one-seventeenth the caloric needs for the first 3 hours and 1/34th of their daily energy requirements for every following half hour in order to achieve a post-prandial steady state (Duez et al., 2008). The Harris Benedict Equation was used to determined total energy needs (based on gender, age, weight, height, and activity factors). Of these calories, 12% were from protein, 44% were from carbohydrates and 44% were from fat.

3.2.3 Biochemical Analysis

Plasma was separated from blood by bench top centrifugation at 2000 rpm for 15 minutes at 4°C. Triglyceride was determined using an enzymatic colorimetric kit (Roche Diagnostics, Mannheim, Germany). Cholesterol was measured with the CHOD-PAP enzymatic colorimetric kit (Roche Diagnostics, Mannheim, Germany). Free fatty acids (FFA) were determined with the NEFA colorimetric method (Wako Industrials, Osaka, Japan). Plasma insulin concentrations were assayed by radioimmunassay utilizing a

human specific insulin kit (Linco Research, St Louis, MO, USA), and glucose was enzymatically determined using a Beckman Glucose Analyzer II (Beckman Instruments Corporation, Fullerton, CA). The homeostasis model assessment for insulin resistance (HOMA-IR) was then derived using fasting insulin and glucose concentrations and used as an index of insulin resistance (Levy et al., 1998).

Triglyceride-rich lipoprotein fractions were separated for each time point through ultracentrifugation at density < 1.006 g/ml after 16 hours, 39000 rpm and at 12°C. Samples were frozen immediately and stored at -80°C. The concentrations of apo B48 in whole plasma samples and TRL fractions were determined by a modified immunoblotting and an enhanced ECL technique described previously (Smith et al., 1997; Vine et al., 2007). Other less intensive methods for measuring the contribution of intestinally-derived lipoproteins (e.g. monoclonal ELISA and RLP-C) are available. Nevertheless, we have elected to apply the western-blot analysis with polyclonal antibodies for greater sensitivity in detecting apo B48 while simultaneously differentiating chylomicrons from apo B100. Briefly, apolipoproteins were separated by SDS-PAGE using 3-8% tris-acetate polyacrylamide gels (Nupage, CA, USA) and then transferred to a PVDF membrane (0.45µm; ImmobilonP^{TM}, Millipore. MA USA). Apo B48 protein was recognized using a commercially available affinity-purified goat polyclonal antibody (Santa Cruz Biotech, CA, Cat#sc-11795). Separated bands were visualized using enhanced chemiluminescence (ECL) (ECL-Advance Amersham Biosciences, UK) and Typhoon Trio imager (GE Healthcare, USA). The mass of apo B48 in both plasma and TRL fractional samples was determined by a linear densitometric comparison (Scion Image) with a standard curve for apo B48.

3.2.4 Statistical Analysis

Subjects were separated into 2 groups based on their fasting plasma insulin concentrations. The median insulin value (48 pmol/L) was used to divide subjects into 'normo-insulinemic' (n = 6, fasting insulin 34.7 ± 2.6 pmol/l, HOMA-IR score 1.2 ± 0.2) and 'hyper-insulinemic' groups (n = 6 fasting plasma insulin 68.8 ±10.8 pmol/l, HOMA-IR score 2.4 ± 0.3). Paired and un-paired t-tests were used to compare physical and metabolic variables across groups, including apo B48 concentrations quantified from whole plasma *versus* TRL fractions and between normo-insulinemic *versus* hyper-insulinemic groups, respectively. Linear regression analysis was used to determine the slope of the area under the curve (AUC) graph between time points 0 and 1 hour. All analyses were performed with Graph Pad Prism 4. Results are presented as mean ± S.E.M. Results were considered significant at a p value < 0.05.

3.3 Results

3.3.1 Biochemical Characteristics

The physical and metabolic characteristics of subjects segregated into hyper-insulinemic and normo-insulinemic groups are presented in Table 3-1. Subjects defined as hyper-insulinemic had significantly elevated fasting plasma insulin concentration ($p = 0.02$) and calculated HOMA-IR score ($p = 0.01$) compared to those in the normo-insulinemic group. All other parameters including BMI, body weight, fasting glucose, FFA levels and fasting plasma lipid levels (total cholesterol, HDL cholesterol and TG) were not different between the hyper-insulinemic and normo-insulinemic groups.

Table 3-1. Physical and fasting plasma metabolic characteristics of normo-insulinemic and hyper-insulinemic subjects

Parameter	Normo-insulinemic (n=6)	Hyper-insulinemic (n=6)	p value
Age (years)	50.2 ± 2.5	44.3 ± 3.7	0.23
Weight (kg)	84.7 ± 5.8	85.5 ± 3.3	0.91
BMI (kg/m^2)	27.7 ± 1.4	28.5 ± 1.0	0.66
Waist Circumference (cm)	99.3 ± 4.4	99.6 ± 3.2	0.96
Insulin (pmol/L)	34.7 ± 2.6	68.8 ± 10.8	0.02
Glucose (mmol/L)	5.0 ± 0.1	4.8 ± 0.2	0.36
Insulin Resistance (HOMA-IR)	1.2 ± 0.2	2.4 ± 0.3	0.01
FFA (mmol/L)	1.2 ± 0.1	1.5 ± 0.3	0.42
Total Cholesterol (mmol/L)	4.9 ± 0.2	4.4 ± 0.2	0.06
HDL Cholesterol (mmol/L)	1.2 ± 0.1	1.2 ± 0.1	0.85
Triglyceride (mmol/L)	0.42 ± 0.04	0.52 ± 0.1	0.35

Values are given as a mean ± S.E.M. p values are by un-paired t-tests.

3.3.2 Fasting Apolipoprotein B48 Concentrations

Fasting apo B48 from whole plasma was significantly greater compared to apo B48 from TRL fractions in both normo-insulinemic and hyper-insulinemic subjects (normo-insulinemic: 10.7 ± 1.2 *versus* 5.2 ± 0.6 µg/ml, p = 0.02; hyper-insulinemic: 16.20 ± 1.96 *versus* 7.08 ± 1.78 µg/ml, p = 0.008).

3.3.3 Apolipoprotein B48 Concentrations in the Fed Steady State

The fed steady-state response, measured by total area under the curve (AUC), is presented in Figure 3-1. Given that the fractional (TRL density < 1.006 g/ml) method of quantifying apo B48 omits smaller particles, we anticipated that a greater concentration of apo B48 would be detected by whole plasma. Predictably, apo B48-AUC from whole plasma was 33% higher compared to apo B48-AUC from TRL fractions among hyper-

insulinemic subjects (418.3 ± 39.2 *versus* 314.5 ± 48.1 µg/ml·17 hrs; p = 0.002). Similarly, determination of apo B48-AUC from whole plasma was 69% greater compared to TRL apo B48-AUC fractions among normo-insulinemic participants (287.8 ± 23.8 *versus* 170.4 ± 8.2 µg/ml·17 hrs, respectively; p = 0.003).

Apo B48 quantified from both whole plasma samples and TRL fractions revealed a significantly greater apo B48-AUC among those with hyper-insulinemia *versus* normo-insulinemia (Table 3-2; p = 0.03 and p = 0.01, respectively).

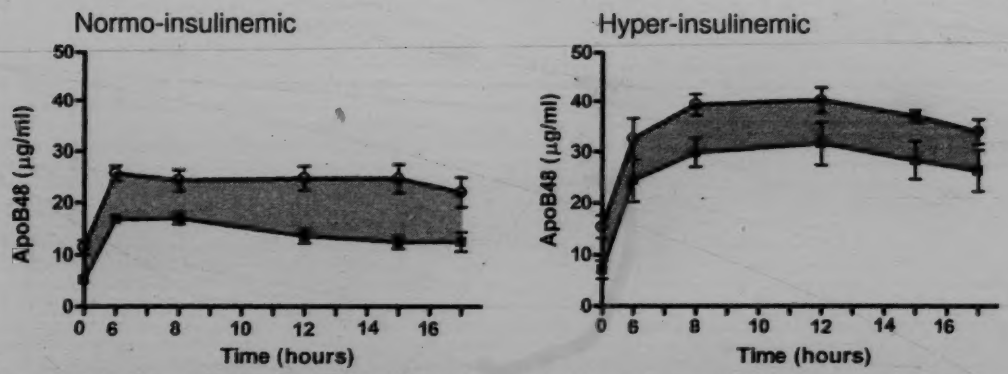

Figure 3-1. The post-prandial associated apo B48 response (AUC) in normo-insulinemic subjects (left panel) and hyper-insulinemic subjects (right panel) during the fed, steady-state. Values are shown for whole plasma (o) and TRL fractions (■) as mean ± S.E.M. Whole plasma found significantly greater total AUC compared to TRL in normoinsulinemic (p = 0.03) and hyperinsulinemic subjects (p = 0.002).

Table 3-2. Apo B48 response in a fed, steady-state among hyper-insulinemic and normo-insulinemic men as determined from whole plasma and TRL fractional samples

Parameter	Normo-insulinemic (n=6)	Hyper-insulinemic (n=6)	p value
Whole Plasma ApoB48 (µg/ml·17 hrs)	287.8 ± 23.8	480.0 ± 70.1	0.03
Fractional ApoB48 (µg/ml·17 hrs)	170.4 ± 8.2	314.5 ± 48.0	0.01

Values are given as a mean ± S.E.M. Hyper-insulinemic subjects showed a significantly greater apo B48-AUC than normo-insulinemic subjects when measured from whole plasma and TRL fractions.

3.3.3 Small Apolipoprotein B48 Remnants in the Fed Steady State

The difference between apo B48 measured in whole plasma and TRL fractional samples was used to further calculate the population of small, apo B48-derived remnant particles (i.e., density > 1.006 g/ml) shown as the shaded region in Figure 3-1. The difference between apo B48 measured in whole plasma and TRL fractional samples was plotted over time during the fed-state in both normo-insulinemic and hyper-insulinemic groups (Figure 3-2). Hyper-insulinemic subjects demonstrated a significantly greater AUC containing apo B48 particles at density > 1.006 g/mL compared to normo-insulinemic controls (125.5 ± 16.9 *versus* 91.3 ± 13.9 µg/ml·17 hrs, respectively; p = 0.03).

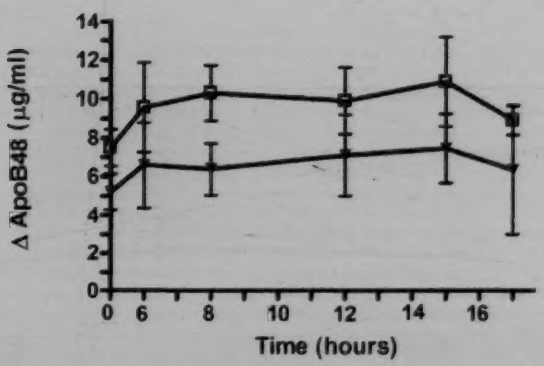

Figure 3-2. Apo B48 remnants in density > 1.006 g/ml. Values are shown for normo-insulinemic subjects (▼) and hyper-insulinemic subjects (□) as mean ± S.E.M. Hyper-insulinemic subjects demonstrated significantly more apo B48 remnant particles (density > 1.006 g/ml) compared to normo-insulinemic participants (p = 0.03).

3.3.4 Apolipoprotein B48 Appearance in Plasma

The slope of the apo B48 AUC curve was also calculated from both the whole plasma and TRL fractional apo B48 AUC graphs between time points 0 (fasting) and 6 hours to represent the chylomicron particle production prior to the fed steady-state (Table 3-3). Subjects in the hyper-insulinemic group showed a greater rate of chylomicron

accumulation when apo B48 was determined from both whole plasma (24.0 ± 4.2 *versus* 14.3 ± 1.0 µg apo B48/ml·6 hrs; p = 0.04) and TRL samples (22.0 ± 4.7 *versus* 11.6 ± 0.8 µg apo B48/ ml·6 hrs; p = 0.03).

Table 3-3. Initial response to feeding from 0 to 6 hours before steady-state is reached in hyper-insulinemic and normo-insulinemic subjects as determined by whole plasma apoB48 and fractional (TRL) methods

Method of apoB48 measurement	Normo-insulinemic (n=6)	Hyper-insulinemic (n=6)	*p* value
Whole Plasma ApoB48 (µg/ml·6 hrs)	14.3 ± 1.0	24.0 ± 4.2	0.04
Fractional ApoB48 (µg/ml·6 hrs)	11.6 ± 0.8	22.0 ± 4.7	0.02

Values are given as a mean ± S.E.M. Greater chylomicron particle accumulation was seen in hyper-insulinemic subjects when determined by both whole plasma and TRL fractions.

3.3.5 Ratio of Apolipoprotein B48 and Triglycerides During the Fed Steady State

The TG:Apo B48 ratio (proportion of TG per particle during the fed steady-state period) was not significantly different between hyper-insulinemic *versus* normo-insulinemic subjects when determined from whole plasma or TRL fractional methods (Figure 3-3). Further, the TG:Apo B48 ratio determined from whole plasma did not differ from TRL fractional methods in hyper-insulinemic participants. However, apo B48 measured from whole plasma detected a greater rate of apo B48-containing chylomicron particles in the TRL fraction compared to whole plasma in normo-insulinemic but not hyper-insulinemic individuals (Figure 3-4; p = 0.02).

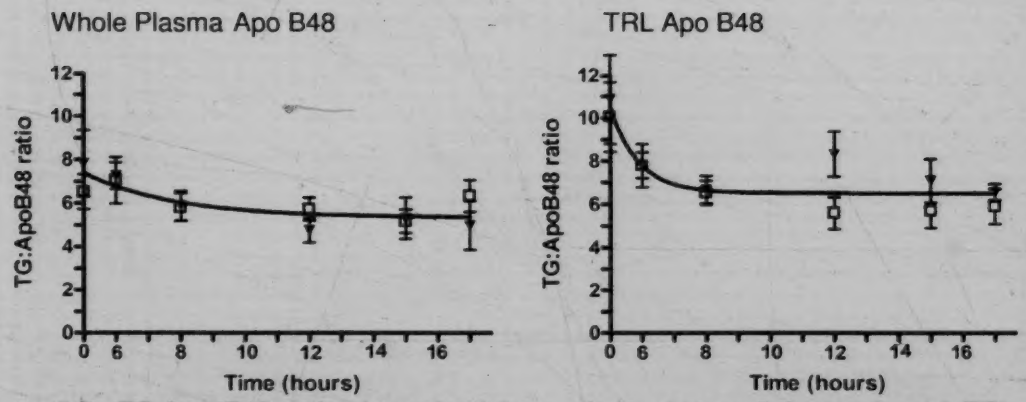

Figure 3-3. TG:Apo B48 ratio determined from whole plasma (left panel) and TRL fractions (right panel). Values are shown for normo-insulinemic subjects (▼) and hyper-insulinemic subjects (□) as mean ± S.E.M.

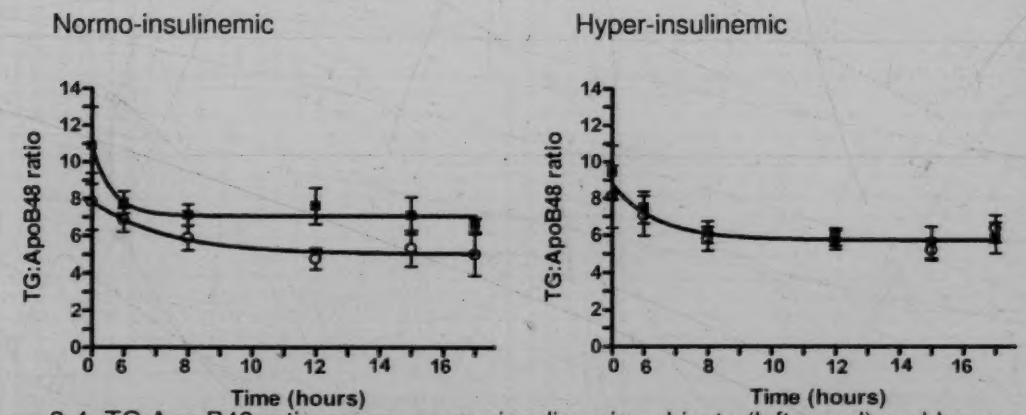

Figure 3-4. TG:Apo B48 ratio among normo-insulinemic subjects (left panel) and hyper-insulinemic subjects (right panel). Values are shown for apo B48 analysis as determined by whole plasma (o) and fractional (TRL) methods (■) as mean ± S.E.M. Measurement of TRL fraction apo B48 in normo-insulinemic subjects revealed larger chylomicrons compared to whole plasma ($p = 0.02$).

3.4 Discussion

The aim of this study was to determine if the preparation of the apo B48 sample effects the identification of dysfunctional post-prandial lipoprotein metabolism, specifically remnant apo B48-containing particles, during the steady-state in subjects with fasting hyper-insulinemia. TRL fractions (density < 1.006 g/ml) have traditionally been used to isolate newly secreted chylomicron particles. Recent evidence has demonstrated that nascent chylomicrons particles are too large to enter the subendothelial space. However, once hydrolyzed by the lipoprotein lipase, chylomicron remnants are able to penetrate the intima (Nordestgaard et al., 1992; Mamo et al., 1994; Mamo et al., 1998; Proctor et al., 2000), impair endothelial function (Doi et al., 1998), and accumulate in the subendothelial space (Mamo et al., 1998; Proctor et al., 2000). In this study, we have found that apo B48 quantified from whole plasma describes a significantly higher apo concentration at fasting and post-prandial, as measured by AUC, compared to measurements from TRL fractions (density < 1.006 g/ml).

Clinical and epidemiology studies have demonstrated that small, dense LDL particles are increased in individuals with insulin resistance (Feingold et al., 1992; Shelby et al., 1993; Austin et al., 1995) and at greater risk of CVD (Gardner et al., 1996; Zambon et al., 1999; St. Pierre et al., 2005). However, the equivalent has not been demonstrated for apo B48-containing particles, despite the knowledge that a larger proportion of intestinally-derived particles reside in fractions > 1.006 g/ml (i.e., smaller remnant particles). We have used the difference in apo B48 concentration from whole plasma and TRL fractions to calculate the contribution of small, apo B48-derived remnant particles (density > 1.006 g/ml). To our knowledge, this is the first study to provide support for the increased presence of apo B48 remnants in density > 1.006 g/ml during a hyper-insulinemic condition.

The mechanisms underlying the link between elevated chylomicron concentrations in insulin resistance and type 2 diabetes mellitus (T2DM) have not yet been fully characterized. We have revealed a greater concentration of small, remnant particles in hyper-insulinemic subjects, which may be the result of impaired clearance or increased intestinal secretion of apo B48-containing chylomicrons. Previous studies have shown a relationship between defective clearance of apo B48 particles due to increased VLDL production in the liver and competition for lipoprotein lipase (LPL) activity (Coppack, 1997; Mälstrom et al., 1997). In the current study, the slope of the apo B48 AUC graph between 0 and 6 hours (representing the accumulation of chylomicron particles) was found to be greater in hyper-insulinemic versus normo-insulinemic subjects when apo B48 is determined either directly from whole plasma or from the TRL fraction. These results are in agreement with recent studies that have demonstrated a higher production rate of intestinally-derived apo B48-containing lipoproteins utilizing a primed-constant infusion of deuterium-labeled leucine in men with hyper-insulinemia and insulin resistance (Duez et al., 2006), as well as T2DM (Hogue et al., 2007).

Measurement of TRL fractional apo B48 in normo-insulinemic subjects revealed larger chylomicrons, with a greater amount of TG per particle, compared to whole plasma. Intuitively, we might expect a similar separation in particle size in hyper-insulinemic subjects. However, we have shown a lack of difference between the TG:Apo B48 curves of whole plasma and TRL fractional measurements, which further supports our observations that individuals with hyper-insulinemia have an increased rate of small, remnant chylomicron secretion during the fed, steady-state.

3.5 Conclusion

In conclusion, we have shown that apo B48 quantified from whole plasma reveals a significantly higher fasting and post-prandial AUC for apo B48 compared to measurements from TRL fractions (density < 1.006 g/ml). We have also found for the first time a significantly greater apo B48 remnant particle (density > 1.006 g/ml) population in men with hyper-insulinemia. These results provide evidence that the measurement of apo B48 in whole plasma provides greater sensitivity to detect small, potentially atherogenic, apo B48-containing particles. Greater accuracy in detecting the production and metabolism of small, apo B48 remnant particles may aid in the diagnosis of post-prandial dyslipidemia and its contributions to CVD-risk during early stages of insulin-resistance.

3.7 References

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Chapter 4: Apolipoprotein B48 Lipemia in Type 1 Diabetes Mellitus

4.1 Introduction

Type 1 diabetes mellitus (T1DM) is associated with a 3 to 4 fold increase in cardiovascular disease (CVD) risk as compared with an age-matched non-diabetic population (Kannel and McGee, 1979). The paradox of T1DM is that despite increased CVD risk, classic fasting lipid indices (low concentrations of high density lipoprotein [HDL] and/or elevated low density lipoprotein [LDL] cholesterol or triglyceride [TG] concentrations) are often comparable to, or better than, those of a healthy population (Howard, 1987). Moreover, increased atherosclerotic risk may not be attributable to other non-lipid risk factors, as there is no increased prevalence of hypertension, obesity, or smoking in this population (Howard, 1987). Currently, the etiology of increased atherosclerotic risk in T1DM is not well understood.

Recent evidence suggests that the metabolic response to multiple meals can exert effects on the initiation and progression of atherogenesis (Tomkin and Owens, 2001). For instance, non-fasting TG has been shown to be a significant predictor of future vascular events in multivariate analysis, and the highest risk was found among those with the greatest non-fasting TG concentrations (Nordestgaard et al., 2007). Furthermore, increased concentrations of intestinally-derived chylomicrons are becoming increasingly acknowledged as an indicator of CVD risk and progression. For instance, impaired metabolism of intestinally-derived remnants have been demonstrated in normolipidemic patients with coronary artery disease (CAD) (Meyer et al., 1996; Weintraub et al., 1996) and other conditions associated with increased vascular disease such as obesity, metabolic syndrome, type 2 diabetes mellitus (T2DM) and familial hypercholesterolemia (Curtin et al., 1996; Mekki et al., 1999; Dane-Stewart et al., 2001).

While the association of impaired remnant metabolism appears to be better defined in dietary and lifestyle-related diseases (obesity, insulin resistance and T2DM), the role of these particles in T1DM is unclear. In T1DM, a limited number of animal studies have observed reduced clearance of chylomicron particles in streptozotocin-induced diabetic rats (Redgrave and Snibson, 1977; Levy et al., 1985). A study of men with T1DM found elevated triglyceride-rich lipoprotein (TRL) (Sf 100-400) concentrations compared to healthy controls (Georgopolous and Phair, 1991). No studies to date have examined apolipoprotein (apo) B48, a specific marker of chylomicron particle number, in the human T1DM population. The advantage of this approach is that apo B48 allows for detection of chylomicrons and chylomicron remnants directly from whole plasma (Mamo et al., 1998). Classically, ultracentrifugation has been used to isolate large, buoyant triglyceride-rich chylomicrons that are newly synthesized from the intestine at a density of < 1.006 g/ml. However, there is now evidence to suggest that smaller apo B48 remnant particles in density fractions > 1.006 are potentially more atherogenic (Mamo et al., 1998), and consequently, may provide a better understanding of CVD risk. In the present study, we examined chylomicron metabolism (via apo B48) in normo-lipidemic men and women with T1DM compared to healthy controls.

4.2 Methods

4.2.1 Participants

Nine normolipidemic subjects with brittle T1DM (5 males, 4 females) and 7 healthy controls (2 males, 5 females) were studied. Subjects were matched based on gender, age (53.3 ± 3.3 vs. 46.5 ± 6.3 years; $p = 0.31$), and BMI (24.9 ± 1.2 vs. 23.7 ± 0.80 kg/m^2; $p = 0.44$). T1DM participants were recruited from the Clinical Islet Transplant Program at the University of Alberta, Edmonton, Alberta. The duration of diabetes in the

nine diabetic subjects was 41.6 ± 3.3 years (range 20-45 years) and glycosylated hemoglobin concentration (Hb_{A1C}) was 8.9 ± 0.51 %. Cholesterol-lowering medications were discontinued one week prior to the study since cholesterol-lowering agents have been shown to alter the clearance of intestinally derived triglyceride-rich lipoproteins (Dane-Stewart et al., 2003). All T1DM patients were treated with a conventional insulin regimen consisting of insulin lispro / aspart with humulin N, humulin R and / or insulin glargine.

Control participants were recruited via advertisements throughout Edmonton. Control subjects were not on any medication and exclusion criteria included smoking, hypertension (>140/90 mmHg), elevated LDL cholesterol (>3.4 mmol/L), low HDL cholesterol (<0.90 mmol/L), and/or a family history of CVD.

All subjects gave informed consent to participate in the study, and the research protocol was approved by the Health Research Ethics Board at the University of Alberta.

4.2.2 Study Protocol

All participants were asked to follow their regular diet and exercise regime leading up to the study. Dietary evaluations, as assessed by 3-day food records, revealed no significant differences in total calories or percent daily calories from fat, carbohydrate, and protein between the study groups (Table 4-1).

Subjects were fasted overnight prior to the test day and a baseline blood sample was obtained from an intravenous catheter inserted into the forearm vein at approximately 8:00 am (0 hours). Breakfast (0.5 hours) and lunch (4.5 hours) were subsequently provided to all participants. Post-prandial blood samples were drawn at 2, 4, 6 and 8 hours into vacutainer tubes containing 0.1% EDTA.

Both breakfast and lunch meals provided approximately 1/3 of daily caloric requirements, with 20%, 30% and 50% of total energy as protein, fat and carbohydrate, respectively. Breakfast consisted of whole wheat toast with margarine, scrambled eggs, fruit and 1% milk. Lunch included pasta with chicken, vegetables in a marinara sauce and 1% milk. These two meals were provided sequentially to represent food consumption in a free-living situation and to elicit a typical post-prandial response. T1DM patients self-administered long-acting insulin dose(s) as prescribed and rapid-acting insulin injections were self-adjusted before or after each test meal.

Table 4-1. 3-day food records of T1DM subjects and matched controls

Macronutrient	Control (n=7)	T1DM (n=9)	p value
Calories (kcal)	1711.2 ± 138.4	1689.3 ± 190.6	0.93
Protein (%)	20.0 ± 1.3	20.7 ± 1.5	0.73
Carbohydrate (%)	54.2 ± 3.8	46.0 ± 2.3	0.07
Fiber (g)	19.6 ± 3.0	19.1 ± 3.2	0.93
Total Fat (%)	25.4 ± 2.3	34.4 ± 3.3	0.06
Saturated Fat (%)	7.6 ± 1.0	11.3 ± 1.6	0.11
Monounsaturated Fat (%)	8.7 ± 1.1	11.0 ± 2.2	0.28
Polyunsaturated Fat (%)	4.5 ± 0.8	5.7 ± 1.0	0.40
Trans Fat (g)	0.7 ± 0.3	0.6 ± 0.5	0.91
n-3 (g)	0.8 ± 0.1	0.7 ± 0.2	0.59
n-6 (g)	5.4 ± 1.2	3.4 ± 1.0	0.21
Cholesterol (mg)	230.0 ± 51.5	180.6 ± 30.8	0.39

Values are given as a mean ± S.E.M.

4.2.3 Biochemical Analysis

Chylomicron concentrations were determined from both whole plasma and triglyceride-rich lipoprotein (TRL) fractions (density < 1.006 g/ml). TRL fractions were separated at each time point by ultracentrifugation (Beckman Optima Centrifuge) at 25,000 RPM for 25 min at 20℃ and were immediately frozen and stored at -80℃. Apo B48 was quantified from whole plasma and TRL fractions (d < 1.006 g/ml) using a previously described SDS-PAGE and modified western blotting technique (Vine et al., 2007). Briefly, apolipoproteins were separated by SDS-PAGE using 3-8% tris-acetate polyacrylamide gels (Nupage, CA, USA) and then transferred to a PVDF membrane (0.45µm; ImmobilonP™, Millipore, MA, USA). Apo B48 lipoproteins were identified using a commercially available affinity-purified goat polyclonal antibody (Santa Cruz Biotech, CA, Cat#sc-11795, Cat#sc-2304). Separated apo B48 bands were visualized with enhanced chemiluminescence (ECL) (Amersham™ ECL Advance™ Western Blotting Detection Kit) and the imaging of proteins was conducted by high sensitivity scanning Typhoon Trio Imager (GE Healthcare, USA). The mass of apo B48 in both whole plasma and TRL fractional samples was quantified by a linear densitometry comparison (Scion Image) from a standard curve of known mass of human apo B48.

Glucose, total cholesterol, LDL cholesterol, HDL cholesterol, and TG were determined by the University of Alberta Hospital laboratory using standard enzymatic colorimetric assays. Insulin was measured using a radioimmunoassay highly specific for human insulin and analogues.

4.2.5 Statistical Analysis

All results are presented as mean ± S.E.M. Absolute post-prandial total cholesterol, TG, insulin, glucose, and apo B48 responses were calculated as area under the post-prandial curve (AUC) (0-8 hours). Incremental post-prandial variations in apo B48 were determined by subtracting the baseline value from the absolute AUC. Statistical comparisons for area and baseline measurements were determined by unpaired t-test. Spearman's correlation coefficients were calculated to study associations. Results were considered significant at a p value < 0.05. All analyses were performed with Graph Pad Prism 4.

4.3 Results

4.3.1 Subject Characteristics

The use of anti-hypertensive medications was more frequent in the group with T1DM. Two subjects with T1DM were prescribed hydrochlorothiazide, 7 used angiotensin converting enzyme inhibitors, 3 used beta blockers, and 2 were prescribed calcium inhibitors.

Eligible participants with T1DM underwent coronary angiography and were found to have one or more lesions which narrowed the lumen diameter by at least 50%. Additional minor disease in multiple vessels was also common among subjects with T1DM. The angiogram results were consistent with accelerated vascular disease associated with diabetes. The control group was not eligible for cardiac catheterization.

Fasting metabolic characteristics of T1DM and control subjects are shown (Table 4-2). Fasting plasma glucose and insulin levels were slightly elevated in participants with T1DM compared to controls, but these differences did not reach statistical significance. The fasting lipid profile (total cholesterol, LDL, HDL and TG) for all subjects was within

the normal range and did not significantly differ between the 2 groups. In contrast, fasting apo B48 was the only lipid-associated parameter found to be higher subjects with T1DM relative to controls (22.8 ± 2.5 *versus* 12.3 ± 0.99 µg/mL, respectively; $p < 0.01$).

Table 4-2. Fasting plasma metabolic characteristics of subjects with T1DM and controls

Parameter	Control (n=7)	T1DM (n=9)	p value
Glucose (mmol/L)	5.0 ± 0.2	7.1 ± 1.1	0.12
Insulin (pmol/L)	6.8 ± 1.9	16.4 ± 11.1	0.06
Total Cholesterol (mmol/L)	4.9 ± 0.2	4.8 ± 0.3	0.58
LDL Cholesterol (mmol/L)	3.0 ± 0.2	2.9 ± 0.3	0.64
HDL Cholesterol (mmol/L)	1.5 ± 0.1	1.5 ± 0.1	0.82
Triglyceride (mmol/L)	0.9 ± 0.1	0.9 ± 0.1	0.69
ApoB48 (µg/mL)	12.3 ± 1.0	22.8 ± 2.5	< 0.01

Values are given as a mean ± S.E.M.

4.3.2 Response of Post-Prandial Insulin and Glucose

Post-prandial insulin and glucose concentrations are presented in Figure 4-1. Concordant with the brittle nature of the subjects' diabetes, participants with T1DM showed a significantly elevated post-prandial glucose AUC (64.4 ± 8.4 *versus* 39.9 ± 1.8 mmol/L·8 hrs; $p < 0.01$) and greater glucose levels at time points 6 and 8 hours ($p = 0.03$ and $p < 0.01$ respectively) following lunch. Insulin AUC did not differ between the 2 groups (71.1 ± 31.1 *versus* 67.1 ± 17.7 mU/L·8 hrs; $p = 0.53$).

4.3.3 Response of Post-Prandial Triglyceride and Cholesterol

Mean values for post-prandial cholesterol and triglyceride concentrations are shown (Figure 4-2). No differences were observed in either TG (8.3 ± 0.8 and 8.9 ± 1.4

mmol/L·8 hrs, respectively; p = 0.66) or cholesterol AUC (38.6 ± 2.0 and 38.7 ± 1.0 mmol/L·8 hrs, respectively; p = 0.96) between T1DM *versus* controls.

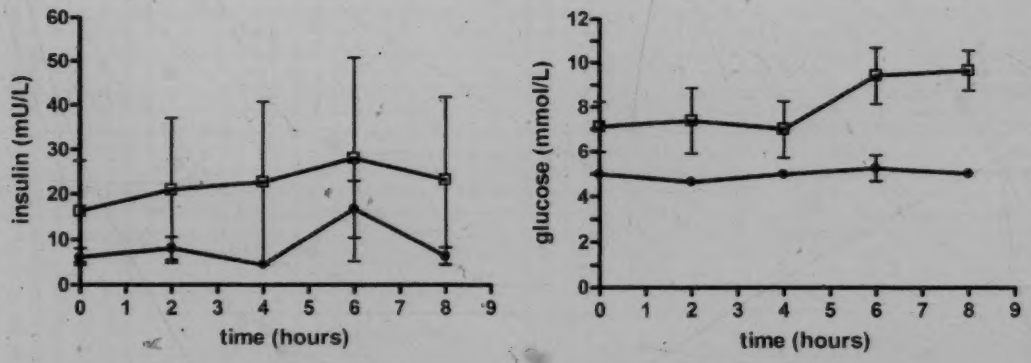

Figure 4-1. The post-prandial response in plasma insulin (left panel) and glucose (right panel) (AUC) for T1DM subjects and controls following the intake of sequential meals. Data are shown for control subjects (•) and T1DM subjects (□) as mean ± S.E.M. Participants with T1DM demonstrated a significantly greater glucose AUC (control: 39.9 ± 1.8 versus type 1 DM: 64.4 ± 8.4 mmol/L·8 hrs; p < 0.01) and glucose levels at time points 6 and 8 hours (p = 0.03 and p < 0.01 respectively) compared to controls.

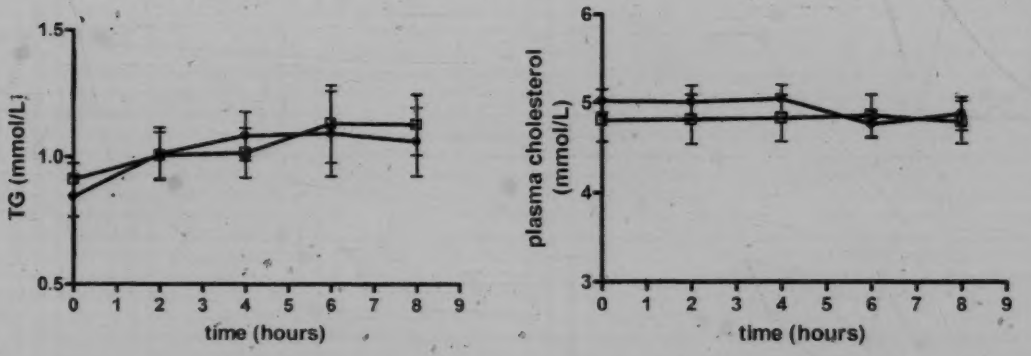

Figure 4-2. The post-prandial response in plasma triglyceride (left panel) and cholesterol (right panel) (AUC) for subjects with T1DM and controls following the intake of sequential meals. Data are shown for control subjects (•) and T1DM subjects (□) as mean ± S.E.M. Values between subject groups are not significantly different.

4.3.4 Post-Prandial Response of Apolipoprotein B48

The post-prandial response of plasma apo B48, as measured by the total area under the curve (AUC), showed that subjects with T1DM had a progressive and significant delay in clearance of remnant particles over the 8 hour time period (Figure 4-3). Total plasma apo B48-AUC indicated that circulating apoB48 mass was 31% greater in subjects with T1DM compared to controls (222.9 ± 11.3 *versus* 169.8 ± 15.87 μg/mL·8 hrs; $p < 0.01$). Ingestion of sequential meals, consistent with a free-living situation, resulted in a biphasic response of plasma apo B48, peaking at 2 hours following the initial meal (i.e., breakfast) (T1DM: 26.1 ± 1.9 *versus* control: 25.5 ± 1.7 μg/mL) and 6 hours after a second meal (i.e., lunch) (T1DM: 33.1 ± 2.2 *versus* control: 22.8 ± 2.2 μg/mL). Apo B48 concentrations did not differ between participants with T1DM *versus* controls at time points 2 and 4 hours following the initial meal (i.e., breakfast). In contrast, participants with T1DM demonstrated circulating apo B48 levels that were 45% greater at 6 hours ($p < 0.01$), which progressively increased to 69% by 8 hours ($p < 0.01$) following a second meal (i.e., lunch) relative to controls.

Corresponding incremental area under the curve (iAUC) showed that the change in apo B48 particles during the post-prandial phase in total (51.81 ± 2.26 and 39.24 ± 1.25 μg/mL·8 hrs; $p = 0.43$) and after ingestion of the first meal (15.87± 2.22 and 21.06 ± 0.99 μg/mL·0-4 hrs; $p = 0.56$) was not different between the 2 groups (data not shown). However, iAUC following intake of a second meal (i.e., lunch) was 5-fold higher in subjects with T1DM compared to controls (25.04 ± 2.23 *versus* 5.03 ± 1.43 μg/mL·4-8 hrs; $p = 0.044$).

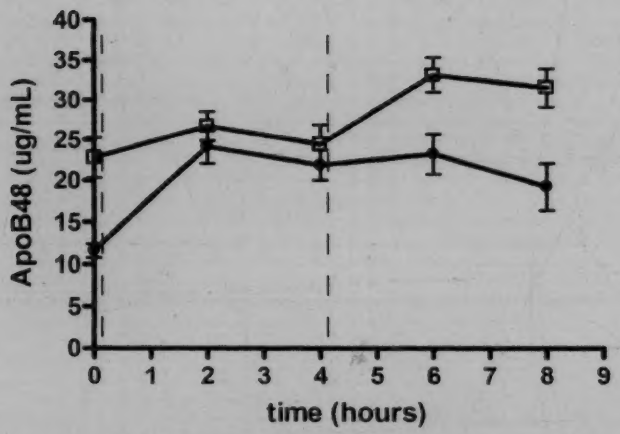

Figure 4-3. The post-prandial associated plasma apo B48 response (AUC) following the intake of sequential meals (represented by dotted lines). Data are shown for control subjects (•) and T1DM (□) as mean ± S.E.M. ApoB48 total AUC (control: 169.8 ± 15.87 versus type 1: 222.9 ± 11.3 µg/ml·8 hrs; $p < 0.01$) and time points 6 and 8 ($p < 0.01$ for both) are significantly elevated in subjects with T1DM compared to the control group.

4.3.5 Comparison to Triglyceride-Rich Lipoprotein Fraction

Apolipoprotein B48 quantified from triglyceride-rich lipoproteins fractions (TRL, density < 1.006 g/ml) showed a 61% greater fasting apoB48 concentration (6.0 ± 1.5 *versus* 2.3 ± 1.2 µg/mL; $p = 0.19$) and a 31% higher apoB48 AUC (59.2 ± 14.3 *versus* 41.1 ± 4.1 µg/mL·8 hrs; $p = 0.58$) in subjects with T1DM compared to controls, but these differences did not reach statistical significance (data not shown). The net mass of apo B48 measured in the TRL fraction was only 30% of the apo B48 determined from whole plasma, and this was true for either T1DM ($p < 0.01$) or control subjects ($p < 0.01$).

4.3.6 Correlation of Plasma Apolipoprotein B48 with Post-Prandial Measurements

The associations between the post-prandial response of plasma apo B48 AUC, insulin AUC, glucose AUC and TG AUC are shown (Table 4-3). Furthermore, no significant associations were observed between apo B48 levels and insulin, glucose and TG concentrations at fasting or any other time point.

Table 4-3. Spearman's correlation coefficients for apolipoprotein B48 and plasma insulin, glucose and triglyceride responses

	r	P
Insulin AUC	-0.3	0.23
Glucose AUC	-0.1	0.80
Triglyceride AUC	0.1	0.68

Values are given as a mean ± S.E.M. No significant associations were observed between apo B48 AUC and insulin AUC, glucose AUC or triglyceride AUC.

4.4 Discussion

The study objective was to examine the metabolism of intestinally-derived chylomicron remnant particles among normolipidemic subjects with T1DM. A significantly higher fasting plasma apo B48 concentration among participants with brittle T1DM compared to matched controls was observed. It is acknowledged that there may be limited power to detect a difference for other indices (fasting lipid profile, glucose, insulin) due to the relatively small number of subjects. Despite the lack of greater statistical power, fasting plasma apo B48 concentration was the only fasting characteristic that differed between subjects with T1DM compared to matched controls. Further, participants with T1DM demonstrated a greater total plasma apo B48 AUC *versus* control subjects. While corresponding total iAUC was not significantly different, iAUC following a second

sequential meal (i.e., lunch) was elevated in subjects with T1DM. Moreover, net AUC provides a more accurate indicator of total cholesterol (associated with the remnant particle) exposure to the arterial wall over time. Thus, we suggest that altered apo B48 metabolism may be associated with increased CVD risk in T1DM, independent of other risk factors such fasting lipid profile, body weight, and age.

Increased plasma apo B48 concentrations in subjects with T1DM were observed at time points 6 and 8 hours and may be attributed to an accumulation of chylomicron particles following a second daily meal. It is important to consider that most individuals are repeatedly in the post-absorptive state, and typically, a third meal would be ingested by individuals over the entire course of a day. It is likely that the addition of a third meal (i.e., dinner) would exacerbate the existing impaired post-prandial apo B48 response.

In contrast to plasma apo B48 responses, fasting and post-prandial apo B48 concentrations from TRL fractions (density < 1.006 g/ml) were not elevated in participants with T1DM. A previous study in humans has reported increased apo B concentration in the TRL fraction of men with well-controlled T1DM compared to controls (Georgopolous and Phair, 1991). The discrepancy between the present study and that reported by Georgopoulos and Phair may be attributed to differences in the individuals selected for study. Also, Georgopoulos and Phair (1991) presented results as total apo B, without distinguishing between the contributions of intestinally-derived apo B48 chylomicrons and hepatically-derived apo B100 particles.

Insulin plays both an indirect and direct role in metabolism of apo B48-containing chylomicrons (Taskinen, 1992). Insulin is an important regulator of VLDL production and is also a key regulator of lipoprotein lipase (Taskinen, 1992). Nevertheless, an association between apo B48 and post-prandial insulin and glucose AUC was not found in the present study. Inter-subject variability in insulin requirements was observed

among T1DM participants studied and may have been a contributing factor to the lack of significance. Lewis and colleagues have examined the post-prandial response in subjects with T1DM following reduced insulin dosages to alter glycemic control (Lewis et al., 1991). Consistent with our findings, Lewis et al. (1991) have suggested that post-prandial chylomicron concentrations are not related to deteriorations in glycaemic control or the level of post-prandial insulin replacement.

The present study was not designed to address mechanisms underlying increased and prolonged residence time of plasma apo B48-containing chylomicrons in T1DM per say. However, some lines of evidence have been proposed. There is some evidence that lipase activity may be decreased among patients with diabetes (De Man et al., 1996). However, if this were the case in the present study, we would expect an increased fasting and post-prandial AUC response for TG and TRL apo B48 (density < 1.006 g/ml). Other investigators have suggested that remnant particle clearance may be impaired in T1DM due to altered particle composition affecting LDL-receptor recognition (Georgopolous and Rosengard, 1989; Georgopolous and Phair, 1991; Taskinen et al., 1992). Indeed, accumulation of apo B48 remnant particles in density fractions > 1.006 g/ml in our study is consistent with impaired apo B48 clearance. Similar conclusions were reached by Georopoulos and Phair (1991) using ^{125}I labeling of apo B particles. Another possible and more novel mechanism that might potentially explain increased accumulation of remnant particles in T1DM is the finding of increased lipid synthesis and/or secretion from the intestine. Studies in streptozotocin-induced diabetic rats have shown both greater intestinal absorption (Young et al., 1983) and increased synthesis (Feingold et al., 1994) of cholesterol. Overproduction of intestinal chylomicrons has recently been demonstrated in humans with insulin resistance (Duez et al., 1996) and

T2DM (Hogue et al., 2007). Thus, future studies should assess the potential impact of increased particle production in T1DM.

4.5 Conclusion

In the fasting and post-prandial state, normolipidemic men and women with brittle T1DM showed significantly elevated plasma apo B48 concentrations, but not from TRL fractions. Furthermore, the increased plasma apo B48 AUC among subjects with T1DM was primarily attributed to the post-prandial response following a second consecutive meal. These differences were observed in the absence of abnormal lipid indices. Therefore, elevated levels of plasma apo B48 could be associated with the increased atherosclerotic risk of brittle T1DM. Further studies utilizing larger sample sizes should examine whether disturbed plasma apo B48 remnants can potentially predict CAD in this population.

4.7 References

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Chapter 5: Collective Discussion and Conclusion

5.1 Collective Discussion

The primary aim of this thesis was to contribute knowledge on how best to measure chylomicron metabolism, either from whole plasma or from a TRL fraction (density < 1.006 g/ml), to provide the most accurate assessment and interpretation of post-prandial lipemia. The results of study 1 (Chapter 3) support part 1 and 2 of our initial hypothesis. First, we confirmed that the detection of apo B48 from whole plasma was statistically greater than density fractions > 1.006 g/ml in a condition of post-prandial lipemia (in this case, hyper-insulinemia). Second, we found that apo B48 quantified from whole plasma revealed a significantly higher AUC for apo B48 compared to TRL fractions (density < 1.006 g/ml) during the fed, steady state. Because whole plasma measurement of apo B48 was a more sensitive measure of chylomicrons than TRL fractions, whole plasma apo B48 was used to examine chylomicron metabolism in a sample of subjects with brittle T1DM. In concordance with part 3 of our hypothesis, fasting and post-prandial AUC for plasma apo B48 was significantly elevated in participants with T1DM compared to matched controls, even in the absence of traditional fasting dyslipidemia.

It is well known that individuals with insulin resistance and T1DM are more likely to have atherosclerosis and its related complications than similar age matched healthy controls, but the etiology is still unclear (Tomkin and Owens, 2001). Although evidence show that improved glycemic control reduces complications related to diabetes (The Diabetes Control and Complications Trial Research Group, 1995), other studies have found that subjects with insulin resistance (without overt T2DM) have more CVD before the development of hyperglycemia (Haffner et al., 2000; Haffner, 2003). Thus, it is not known whether hyperglycemia, lack of insulin action and / or other metabolic

derangements exclusive of hyperglycemia are atherogenic (Goldberg and Dansky, 2006). In our selected study populations of hyper-insulinemia / insulin resistance and T1DM, no commonalities existed in relation to glycemic control, plasma insulin concentrations and disease presentation. However, post-prandial lipemia was observed in both subject with hyper-insulinemia and T1DM compared to controls.

The results of this thesis suggest that both groups of hyper-insulinemic and T1DM participants exhibited elevated concentrations of small chylomicron remnants in density fractions > 1.006 g/ml. A decrease in the size and an increase in density of LDL cholesterol are characteristics of insulin resistance and T2DM (Goldberg, 2001). *In vitro*, small dense LDL particles are more readily oxidized, and these particles can display an impaired interaction with LDL receptors (Goldberg, 2001). Several human studies also imply that small dense LDL particles are an additional marker for atherosclerotic development (Gardner et al., 1996). However, an equivalent body of evidence does not exist for apo B48-containing chylomicron particles, despite the knowledge that a larger proportion of intestinally-derived particles reside in fractions > 1.006 g/ml (i.e., smaller remnant particles). Additional studies are needed to assess the affect of chylomicron particle size and density in subjects with hyper-insulinemia and T1DM.

Chapter 2 (Section 2.3.1.6) reviewed the mechanisms underlying abnormal chylomicron metabolism in hyper-insulinemia / insulin resistance. The mechanisms accounting for increased chylomicron accumulation and / or decreased particle clearance in T1DM are currently not known, and the following expands upon the lines of evidence that have been proposed. It has been hypothesized that chylomicron overaccumulation may be the indirect result of increased hepatic VLDL release. In poorly controlled patients with T1DM, the antilipolytic effect of insulin may be diminished, resulting in higher free fatty acid flux from adipose tissue and increasing substrate availability for hepatic VLDL

production (Jensen et al., 1989). Consequently, the sudden increase in chylomicron particles following a meal may compete with VLDL for LPL activity leading to chylomicron accumulation in the circulation.

Similar to insulin resistance, elevated post-prandial chylomicron concentrations in T1DM may directly result from decreased LPL activity (De Man et al., 1996). Another possible mechanism for elevated apo B48 concentrations in T1DM involves increased lipid absorption from the intestine. Studies in streptozotocin-induced diabetic rats found a greater intestinal absorption (Young et al., 1982) and synthesis (Feingold et al., 1994) of cholesterol compared to controls. Furthermore, studies utilizing compositional analysis have shown that apo B particles from the TRL fraction of men and women with T1DM are cholesterol-enriched compared to matched controls (Georgopoulos and Rosengard., 1989; Georgopoulos and Phair., 1991). Compositional abnormalities may explain the decreased clearance of post-prandial chylomicrons in T1DM. A reduced hepatic uptake of LDL particles from patients with T2DM has been associated with an altered lipid composition of the LDL particle (Taskinen, 1992). It is possible that compositional alterations in chylomicrons would also cause changes in the binding of these particles to their receptors. Moreover, rat studies using lipid emulsions as a model for the metabolism of TG-rich lipoproteins have shown that the accumulation of these particles is due to impaired tissue clearance (Redgrave and Callow, 1990).

In Chapter 4 of this thesis, subjects with T1DM had poor glycemic control. Hyperglycemia contributes to macrovascular complications (Brownlee 2001; Reusch 2003; Goldberg and Dansky 2007), and it is possible that elevated glucose levels may confer additional risk and / or exacerbate existing post-prandial dyslipidemia among the T1DM participants studied (Ebara et al., 2000). Large prospective clinical studies suggest a strong correlation between glycemia, atherosclerotic plaque burden and

cardiovascular events (Laakso, 1996; Tominaga et al., 1999; Ledru et al., 2001). Four major molecular signaling pathways have been implicated in the hyperglycemic injury of endothelial cells and other cell types (Brownlee 2001). These include: increased polyol pathway flux; increased advanced glycation end-production formation; activation of protein kinase C isoforms; and increased hexosamine pathway flux (Brownlee 2001). Nishikawa et al. (2000) have proposed a unifying mechanism that integrates the four different pathogenic mechanisms through a single hyperglycemia-induced process: overproduction of superoxide by the mitochondrial electron transport chain. Many studies have previously shown that hyperglycemia increases oxidative stress (Giugliano et al., 1996), but neither the underlying mechanism nor the consequences for other pathways of hyperglycemic damage were known.

5.2 Future Directions

A number of unanswered questions from this thesis provide additional avenues for further study. For instance, no published reports have compared the methodological differences between the use of immunoblotting and ELISA among individuals who are at increased risk of atherosclerosis compared to a healthy population. Both immunoblotting and ELISAs are capable of detecting apo B48 from whole plasma but differ in their level of sensitivity, time, and cost. In order to extend chylomicron detection to a clinical setting, investigations into a standard method of measurement, with established reference values indicating level of risk for various populations, should be further explored.

5.3 Collective Conclusion

In conclusion, the measurement of apo B48 from whole plasma provides greater sensitivity to detect small, potentially atherogenic apo B48-containing remnant particles in both the hyper-insulinemic and T1DM populations. Furthermore, the determination of apo B48 from whole plasma detected differences in chylomicron metabolism between subjects with brittle T1DM and healthy controls. Collectively, the studies of this thesis suggest that greater accuracy in detecting the metabolism of chylomicron remnants can impact the interpretation of post-prandial lipemia and its contributions to CVD risk.

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